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The role of the transcription factor LEF-1 in lymphocyte differentiation

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THE ROLE OF THE TRANSCRIPTION FACTOR LEF-1 IN LYMPHOCYTE DIFFERENTIATION

by

Ross Masao Okamura

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Microbiology

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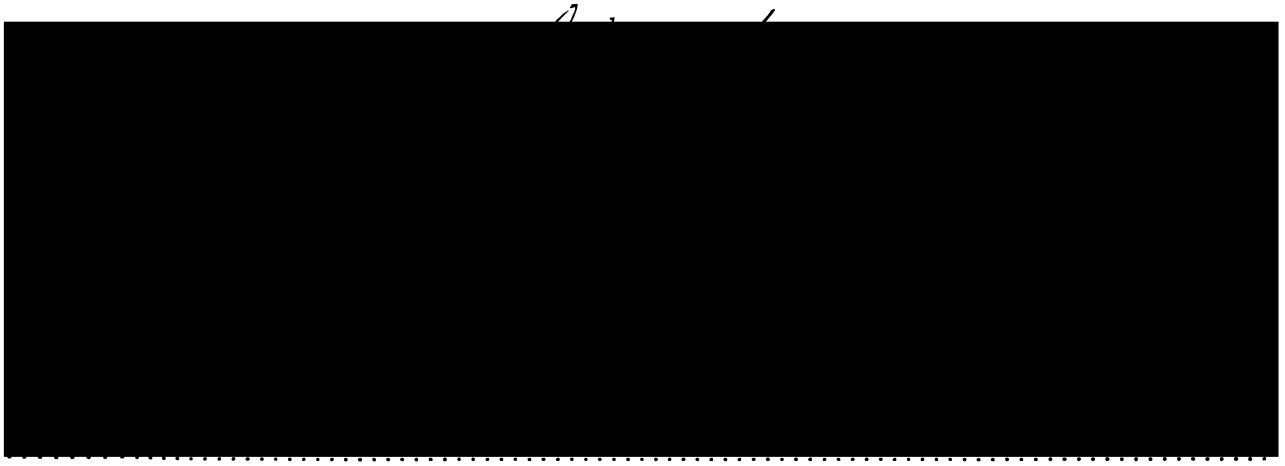

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This thesis is dedicated to Dennis and Mary Okamura, my parents.

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Contribution to Described Work

All of the data presented in the figures in this manuscript was produced by myself, with the exception of figure 2-1A. Figures 3-6 and 3-7 were produced in collaboration with Mikael Sigvardsson and Juan Galceran, respectively. Chapter Two of this manuscript contains data (figures 2-1 and 2-2) which were originally published in Genes and Development, C. van Genderen, R.M. Okamura, I. Farinas, R.-G. Quo, T. Parslow, L. Bruhn, R. Grosschedl, (1994) 8:2691. Chapter Three of this manuscript is a reprint of the material as it appears in *Immunity*, R.M. Okamura, M. Sigvardsson, J. Galceran, S. Verbeek, H. Clevers and R. Grosschedl, (1998) 8:11-20.

The role of the transcription factor LEF-1 in lymphocyte differentiation.

Ross Masao Okamura

Abstract

The differentiation of B and T lymphocytes is regulated by a complex combination of extracellular and intracellular processes. To gain an understanding of the mechanisms used during lymphocyte differentiation, lymphocyte-specific regulators of transcription have been isolated and characterized. One such transcription factor, Lymphoid Enhancer Factor-1 (LEF-1) is expressed during both B and T lymphocyte differentiation. LEF-1 is a member of the HMG family of transcription factors and is able to bind the specific DNA sequence WWCAAAG with its HMG domain. To determine the targets of regulation of LEF-1 within the lymphocyte lineage, we generated a mouse with a targeted disruption of LEF-1. LEF-1 is expressed during B lymphocyte differentiation within the pro-B cell stages but not at later mature stages. The production of BP-1, MHC class II, and TdT are altered in LEF-1-deficient B cells, however this does not prevent the differentiation of mature, functional B cells. A non-conventional population of B cells, B1a cells, were found to require LEF-1 but this requirement appears to be a strain-specific. Within the T cell lineage, LEF-1 is expressed in varying degrees during T lymphocyte differentiation, with the highest levels coinciding with the production of the T cell receptor alpha (TCRα) gene, a target of transcriptional regulation of LEF-1. Analysis of LEF-1-deficient T cells for the expression of TCRα revealed no absolute requirement for LEF-1. Additionally, T lymphocyte differentiation and function were unimpaired in LEF-1-deficient mice. An explanation for this result was a potential

functional redundancy with another transcription factor. To explore this possibility, we crossed LEF-1-deficient mice with mice deficient for the expression of the related HMG family member TCF-1. In the absence of both genes, a complete block in differentiation not seen in either single-deficient mouse was observed at the immature $CD8^+$ single positive stage of differentiation. Furthermore, an incomplete block at the $CD25^+CD44^-$ stage of differentiation was detected. Finally, the TCRα gene was not expressed within the double-deficient mouse, demonstrating that LEF-1 and TCF-1 regulate the transcription of this gene in a redundant manner. Taken together, these data describe the role of the transcription factor LEF-1 during lymphocyte differentiation.

Arthur Weiss, M.D., Ph.D.

Thesis committee chairman

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Chapter 1

Introduction

Lymphocyte Differentiation

The mammalian immune system is an intricate defense network of finely selected cells, specifically designed to protect the body from an innumerable variety of threats. The production a competent immune repertoire, which is able to distinguish self from non-self, involves a strictly regulated process that is composed of a series of complex checkpoints. B and T lymphocytes, which are the subjects of this rigorous selection process, are derived from multi-potential stem cells influenced by environmental influences. During embryonic development, hematopoetic stem cells are localized in the fetal liver and, after birth, migrate first to the spleen, and then finally to the bone marrow. Within these tissues, stromal cells provide conditions supportive of differentiation to numerous hematopoetic lineages. The multi-potential stem cells found in this environment can then become any of a wide variety of myeloid or lymphoid cells depending on the differentiation program that is selected (figure 1-1).

Cells that are destined to become B lymphocytes differentiate within the bone marrow in the adult immune system. The maturity of the B cell can be determined by the sequential expression of cell surface antigens as well as by the successful production of the B cell antigen receptor. While the order of the stages of B cell differentiation are well agreed upon, the nomenclature for these cell populations is diverse (figure 1-2). Our lab has chosen to use the nomenclature described by Richard Hardy [1] for identifying the stages of B cell differentiation and for consistency, only this system is used within this manuscript. The earliest pro-B cell stages (fractions A, B, and C) can be distinguished from more mature populations by the expression of the surface antigen CD43. The expression of BP-1, λ5 and heat-stable antigen (HSA) can be used to further

Figure 1-1 Origin of the cells of the immune system. The various types of mature cells listed on the right are derived from a single type of pluripotential stem cell. The exact steps of hematopoetic differentiation are not all known and in some cases, such as for the natural killer cells and dendritic cells, even the intermediate progenitors are unidentified.

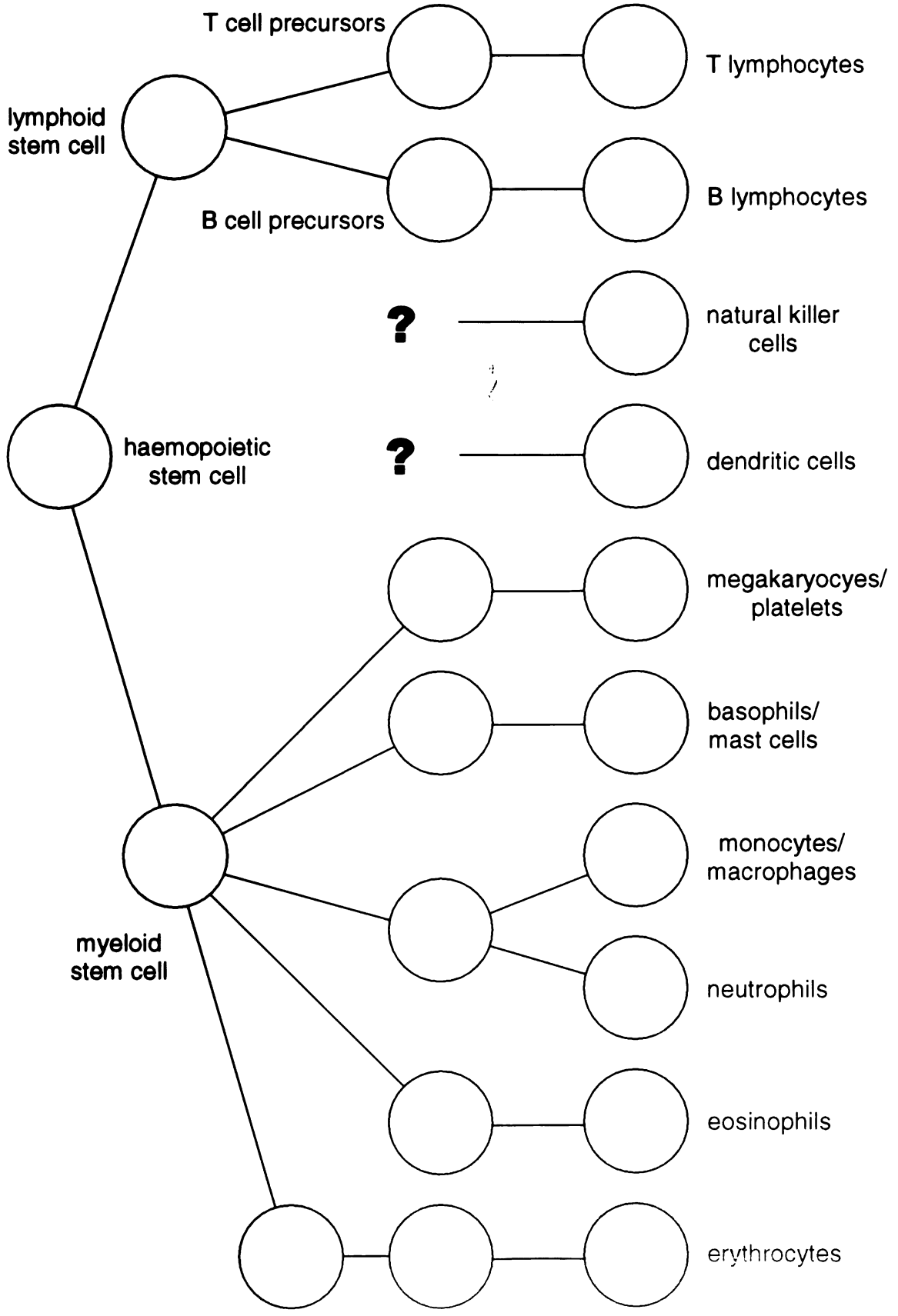
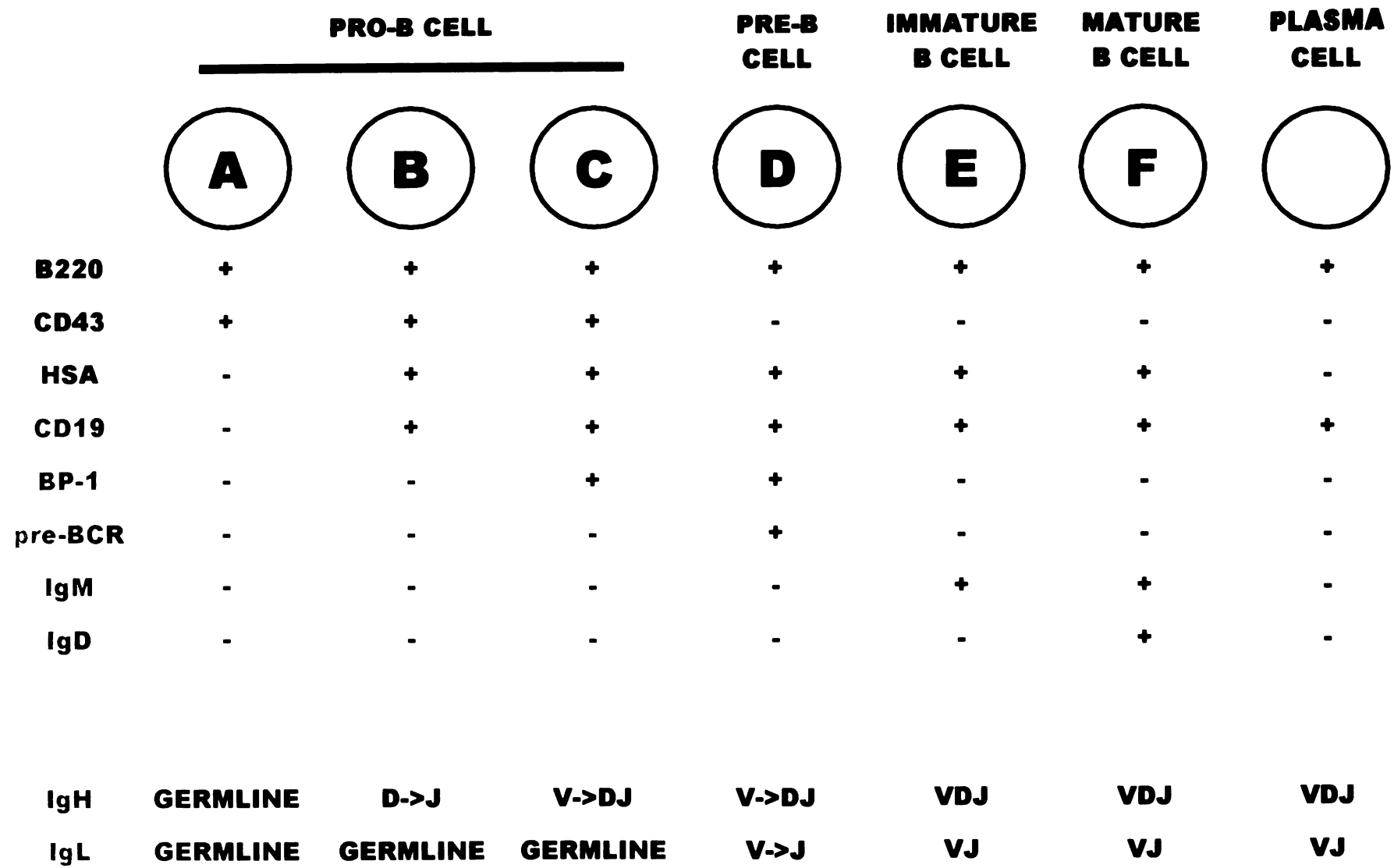

T cell precursors
T lymphocytes
lymphoid stem cell
B cell precursors
B lymphocytes
?
natural killer cells
haemopoietic stem cell
?
dendritic cells
megakaryocyes/ platelets
basophils/ mast cells
monocytes/ macrophages
myeloid stem cell
neutrophils
eosinophils
erythrocytes

Figure 1-2 Murine B cell differentiation. The classification of the various stages is defined primarily by the absence (-) or presence (+) of specific surface markers as described by Hardy [1]. The rearrangement of the immunoglobulin heavy (IgH) and light (IgL) chains are indicated in the lower half below each stage of differentiation.


PRO-B CELL
PRE-B CELL
IMMATURE B CELL
MATURE B CELL
PLASMA CELL
A
B
C
D
E
F
B220 + + + + + + +
CD43 + + + - - - -
HSA - + + + + + -
CD19 - + + + + + +
BP-1 - - + + - - -
pre-BCR - - - + - - -
IgM - - - - + + -
IgD - - - - - + -
IgH GERMLINE D->J V->DJ V->DJ VDJ VDJ VDJ
IgL GERMLINE GERMLINE GERMLINE V->J VJ VJ VJ

sub-divide the pro-B cell stage [2, 3]. During these developmental stages, the immunoglobulin heavy chain (IgH) locus undergoes DNA rearrangement (figure 1-3), first recombining D_H to J_H, and then V_H to DJ_H. The defined fractions can be used to identify the populations in which this recombination occurs, with D_H to J_H beginning in fraction A and V_H to DJ_H starting in fraction C (figure 1-2). Within the next step in differentiation, the pre-B cell (fraction D), expression of CD43 is lost, the cells become smaller in size and the IgH is productively rearranged. Surface expression of IgH in complex with the molecules Igα and Igβ, serves to promote both allelic exclusion of the IgH locus [4-6] and the activation of transcription and rearrangement of the immunoglobulin light (IgL) chain locus [7-10]. The production of a rearranged IgH:IgL receptor complex on the surface of the B cell signals the differentiation to the mature B cell stage and the cells begin to display homing receptors [11] that allow them to migrate to the peripheral lymphoid organs.

Precursors for T lymphocytes also appear first in the bone marrow, but then migrate to the thymus, where they seed themselves. The thymus itself is composed of numerous lobules, which can be subdivided into an outer cortex and inner medulla. During differentiation, T cells move from the cortex to the medulla and finally leave the thymus upon selection and maturation. As with the B lymphocytes, the maturational status of T lymphocytes can also be identified by the tightly regulated expression of surface antigens and the selection of a functional T cell antigen receptor. Unlike B cells, there is no argument as to the nomenclature used to describe the different stages of T cell differentiation (figure 1-4). The earliest committed T cell precursors that migrate into the thymus are negative for most T cell surface antigens but express low levels of CD4 [12].

Figure 1-3 Immunoglobulin heavy chain rearrangement. The process of rearrangement of the immunoglobulin heavy chain occurs in roughly two steps, starting from an unrearranged, germline locus. In the mouse, the immunoglobulin heavy chain locus contains roughly 10^2-10^3 Vh, 10-20 Dh, and 4 Jh regions. Selected Vh families are listed in order of their proximity to the Dh regions. In the first step of rearrangement, a single Dh region and a single Jh region are selected and the intervening genomic DNA is excised by the RAG recombination complex. After this has been successfully completed, a second rearrangement step is mediated by the same recombination machinery. The genomic DNA between one Vh region and the DhJh product is removed producing a final V(D)J rearrangement. The exact mechanism of selecting the precise Vh, Dh, or Jh regions used is unknown, but a preference for Dh proximal Vh regions is seen in fetal liver immunoglobulin heavy chain rearrangements.

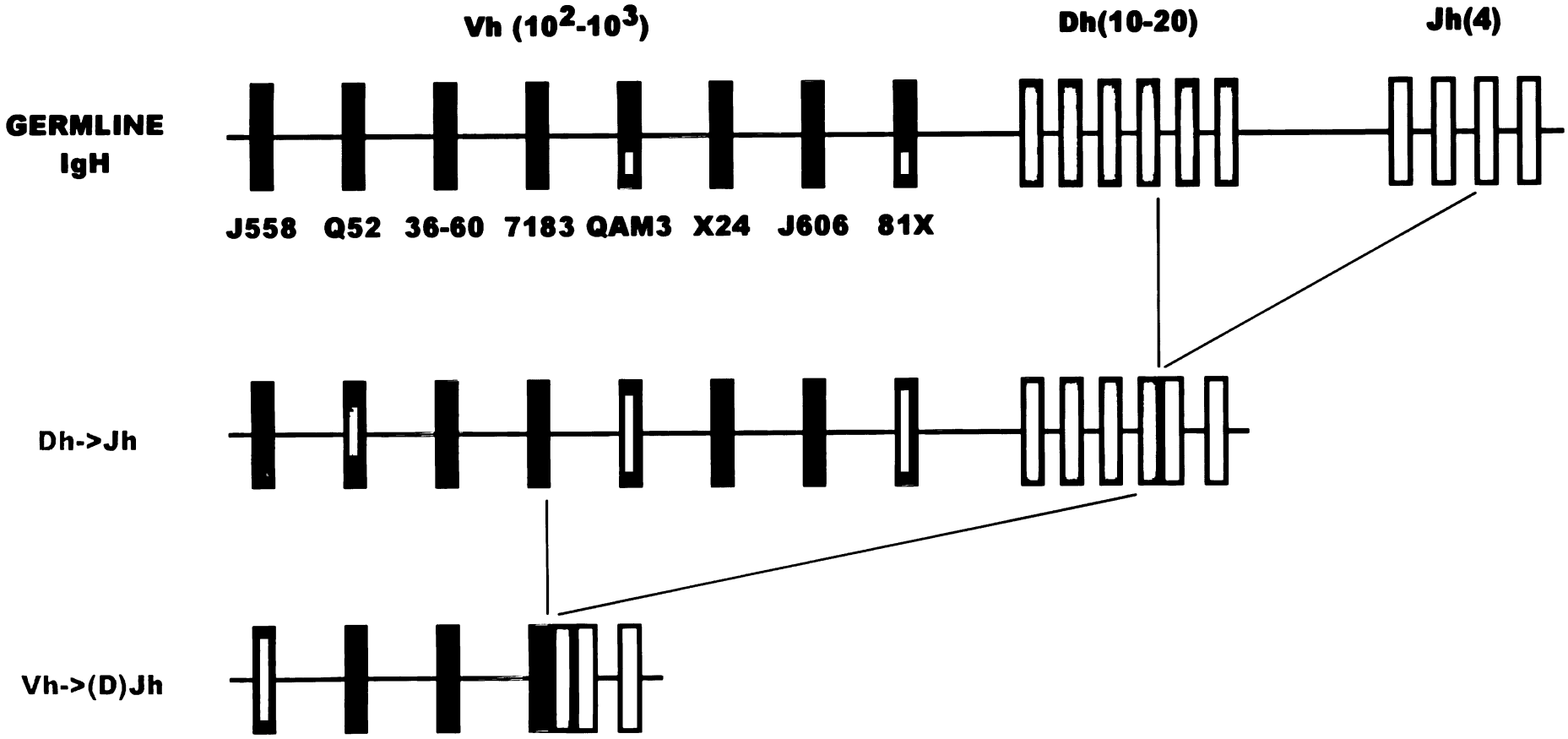
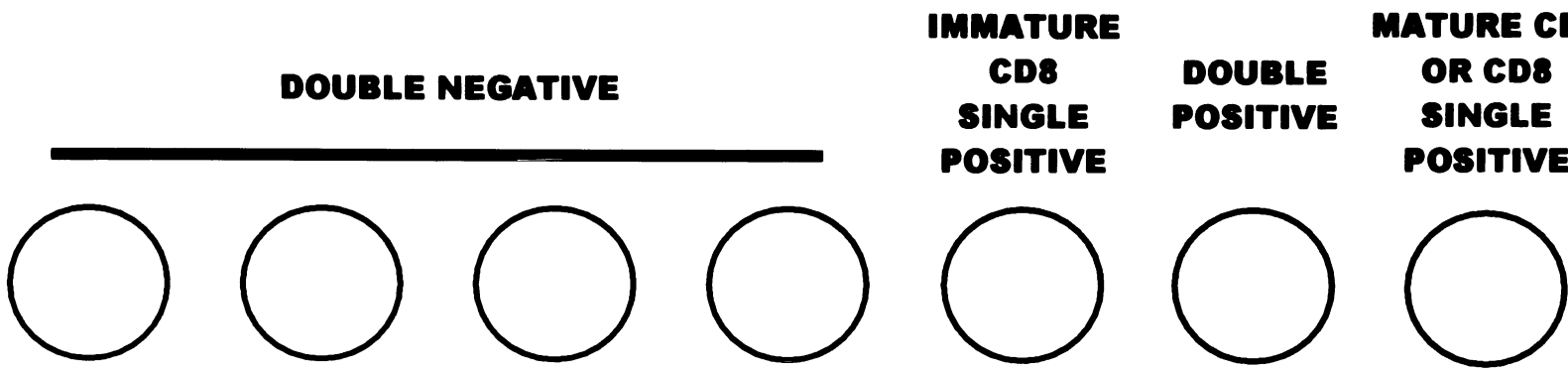

Vh (10^2-10^3)
Dh(10-20)
Jh(4)
GERMLINE
IgH
J558
Q52
36-60
7183
QAM3
X24
J606
81X
Dh->Jh
Vh->(D)Jh

Figure 1-4 Murine α/β T cell differentiation. The classification of the various stages is defined primarily by the absence (-) or presence (+) of specific surface markers. Major checkpoints in differentiation are present at the third double negative (DN) stage ($CD44^-$ /$CD25^+$) where a functional pre-T cell receptor (pTα/TCRβ/CD3) is required, and at the double positive stage (DP), where the TCRα/TCRβ/CD3 complex is necessary for T cell selection.



CD44	+	+	-	-	-	-	-
CD25	-	+	+	-	-	-	-
CD3	-	low	low	low	low	+	+
CD8	-	-	-	-	+	+	+/-
CD4	-	-	-	-	-	+	-/+
pTα	-	+	+	+	-	-	-
TCRβ	-	-	+	+	+	+	+
TCRα	-	-	-	-	+	+	+

Differentiation of these precursors generates thymocytes that are negative for surface expression of both CD4 and CD8 (double negative, DN). These cells include four distinct differentiation stages that are defined by ordered changes in the surface expression of CD44 and CD25 (reviewed in [13]). During the third DN stage ($CD44^-$ $CD25^+$) of thymocyte differentiation, expression of the *Rag1* and *Rag2* genes is upregulated and the genes encoding the T cell receptor (TCR) β, δ, and γ chains are rearranged [14]. Proteins produced from productively rearranged TCRβ genes associate with pre-TCRα (pTα) chains, generating a pre-T cell receptor complex [15]. Targeted disruptions of the TCRβ and pTα genes have shown that the pre-TCR complex is important for the generation of α/β T cells as differentiation in these mice is blocked to varying degrees of completion at the $CD44^-CD25^+$ DN stage of differentiation [16, 17]. Cells that fail to express a functional pre-TCR complex, but productively rearrange TCRδ and γ genes can alternatively differentiate into cells that are referred to as γ/δ T cells (reviewed in [18]). Differentiation of TCRα/β-expressing cells continues with the appearance of CD8 on the cell surface, producing cells termed immature $CD8^+$ single positive cells (ISP). These cells differentiate into $CD4^+CD8^+$ double positive cells (DP) which again upregulate the *Rag1* and *Rag2* genes and rearrange the TCRα locus. Finally, the double positive cells undergo selection to produce either $CD4^+CD8^-$ or $CD4^-CD8^+$ mature single positive cells (MSP).

Lymphocyte-specific transcription factors

The process of differentiation is controlled by the regulation of transcription that occurs within the cell. General transcription machinery is used in all cell types and is

evolutionarily conserved across species. To allow for different cellular fates, a mechanism to dictate specific patterns of gene expression is required. This is accomplished within the nucleus by the regulation of accessibility of the DNA and by the expression of tissue-specific transcription factors. The identification and characterization of lymphocyte-specific transcription factors is thus one way to elucidate the intricate mechanisms by which B and T cell differentiation occurs. To accomplish this, two main strategies have been used. The first and more satisfying because of its completeness, is the isolation of proteins that bind to regulatory regions of lymphocyte-specific genes. This method provides both the transcription factor and the target gene it regulates. However, since not all of the regulatory regions of all of the genes essential for lymphocyte differentiation have been determined, a more blunt approach has been necessary to find other essential transcription factors. To screen for novel transcription factors, it is possible to obtain pure populations of the different stages of lymphocyte differentiation to compare and contrast for genes that are differentially expressed. Likely candidates can then be screened for the ability to bind DNA and regulate transcription.

Attempts to identify the regulatory elements involved in lymphocyte lineage commitment has led to the discovery of a host of transcription factors (reviewed in [19, 20]), some of which fulfill this critical role and others which are expressed, but control an essential step at a somewhat later stage. In analyzing the types of transcription factors that have been cloned, certain themes of protein structure are apparent, allowing the establishment of families of transcription factors which are related by their DNA-binding domains, but differ in their expression pattern and in their ability to either repress or activate transcription. One such family of proteins is the high-mobility group (HMG)

family of proteins, which are characterized by a conserved DNA-binding motif termed the HMG domain (reviewed in [21]). While not all HMG proteins are lymphocyte-specific, several have been grouped into a subfamily of proteins called the LEF/TCF family because of their nearly identical HMG domains. The subject of this dissertation is the analysis of one member, LEF-1, and its role in the regulation of lymphocyte differentiation.

Lymphoid Enhancer Factor-1

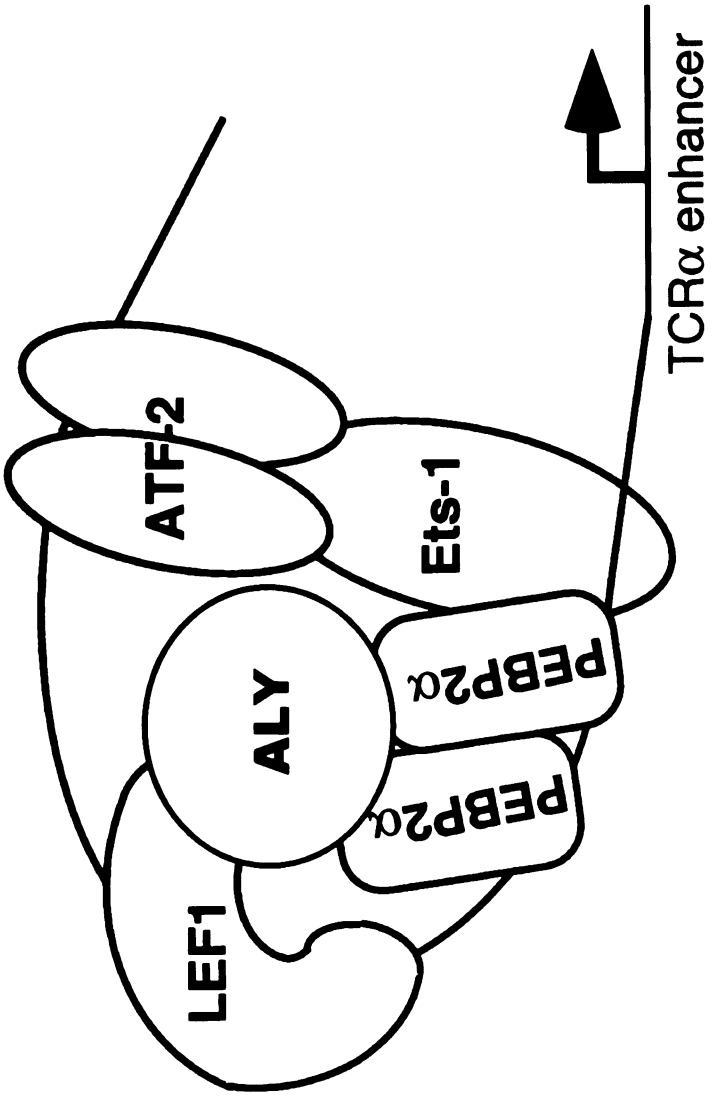
Lymphoid Enhancer Factor 1 (LEF-1) was cloned initially as a pre-B and T lymphoid-specific gene encoding a DNA-binding protein of the family of high mobility group (HMG) proteins [22, 23]. Interestingly, LEF-1 was identified independently by both previously mentioned techniques for isolating transcription factors, with our laboratory cloning LEF-1 through a differential screen [22] and Kathy Jones' laboratory identifying LEF-1 based on its DNA-binding ability [23]. LEF-1 is encoded by a gene located on chromosome 3 in the mouse and chromosome 4 in humans [24]. Sequence-specific recognition of DNA by LEF-1 protein was found to be governed by an 85-amino acid region, termed the HMG domain, that displays sequence homology with other members of this family of proteins [25]. Functional and biochemical characterization of LEF-1 indicated that this protein participates in the regulation of the enhancer associated with the TCRα gene [22, 23]. LEF-1 protein has the capacity to induce a sharp bend in the DNA helix [26, 27] and is dependent on other enhancer-bound proteins to activate transcription [28, 29]. Together with the requirement for a particular arrangement of factor-binding sites in the TCRα enhancer, these observations were interpreted to suggest

an "architectural" role for LEF-1 in the assembly of a higher order nucleoprotein complex [30] (figure 1-5). In addition, LEF-1 contains a transcriptional activation domain that is enhancer context-specific [31, 32].

LEF-1 has been shown to be able to form at least two different multiprotein complexes in order to regulate transcription. In an effort to determine the role of the context-dependent activation domain, Laurakay Bruhn used a yeast two-hybrid assay to isolate the transcription factor ALY [33]. ALY was characterized as binding to LEF-1 via the context-dependent activation domain as well as to the transcription factor AML-1, placing ALY potentially within the multiprotein complex formed on the TCRα enhancer (figure 1-5). ALY is unable to activate transcription alone, but is required for *in vitro* TCRα enhancer function [33].

A second multiprotein complex in which LEF-1 can regulate transcription was identified when it was determined that LEF-1 is able to interact through its amino terminus with β-catenin in response to Wnt/Wingless signaling [34, 35]. This provides a mechanism by which extracellular interactions could regulate the transcriptional activation activity of LEF-1. While Wnt signaling may influence LEF-1 proteins in other tissues, there is no conclusive evidence in lymphocytes that either LEF-1 or TCF-1 requires an interaction with β-catenin to regulate transcription. A truncated form of LEF-1 lacking the β-catenin interaction domain has been shown *in vitro* to activate the TCRα enhancer equally as well as full-length LEF-1 protein [29]. This would indicate that the regulation of this target gene is independent of a Wnt signaling pathway.

Figure 1-5 The TCRα enhancer is regulated by the transcription factor LEF-1. The TCRα enhancer is represented as a loop of DNA, which is bound at sequence specific binding sites by the transcription factors LEF-1, PEBP2α, Ets-1 and ATF-2. The HMG domain of LEF-1 is responsible for both DNA-binding and inducing the bend in the DNA such that ATF-2 and Ets-1 are able to complete a multiprotein enhancer complex. The transcriptional co-factor ALY is unable to bind DNA, but can associate with LEF-1 and PEBP2α through protein:protein interactions. All of the represented transcription factors are required for *in vitro* transcriptional activation of the TCRα enhancer.

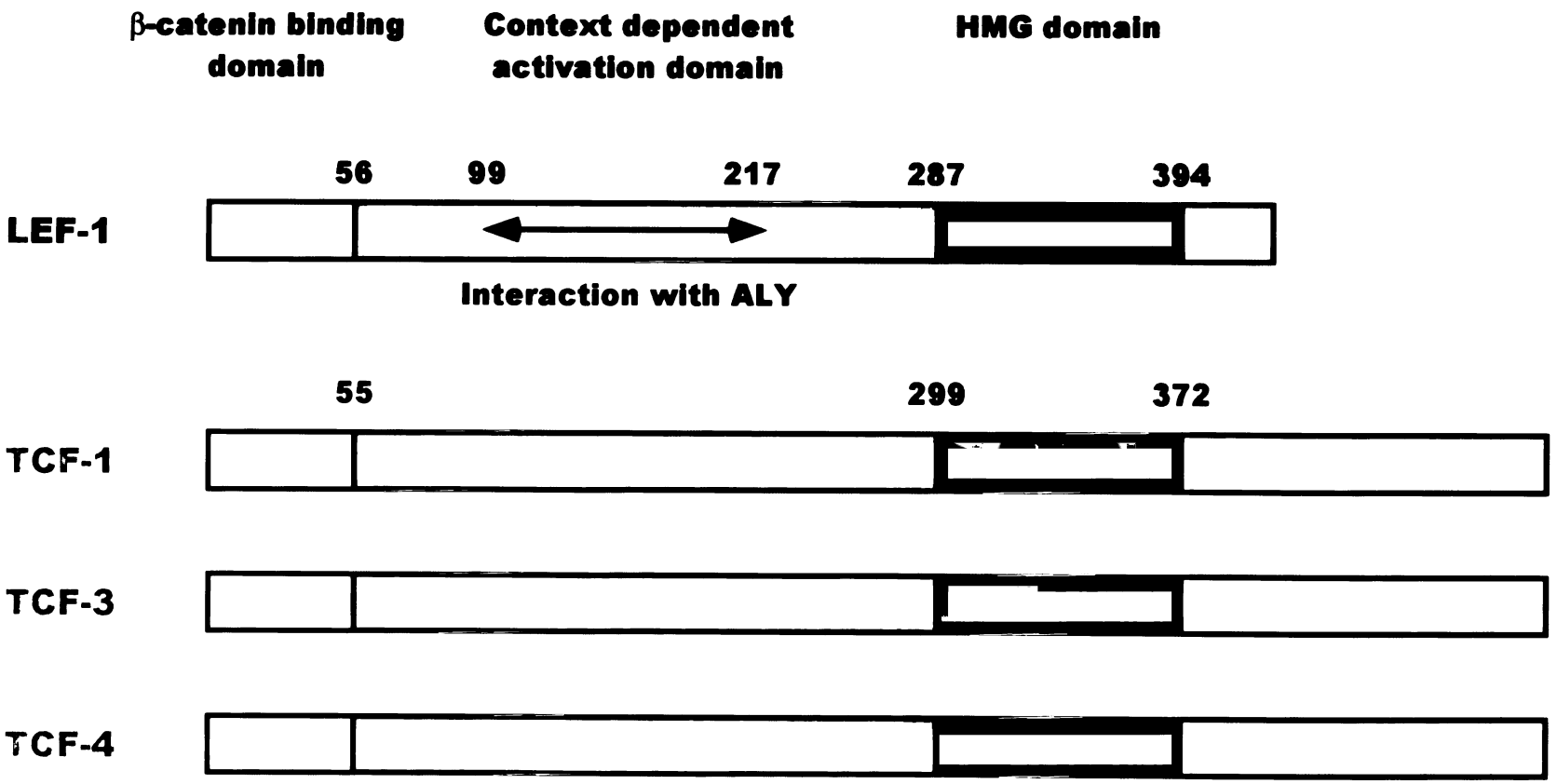

TCRα enhancer
ATF-2
Ets-1
PEBP2α
PEBP2α
ALY
LEF1

The expression pattern of LEF-1 in mice was determined by RNA analysis of adult tissues and by *in situ* hybridization of embryo sections [22, 36, 37]. In the adult mouse, RNA expression of LEF-1 is restricted to the thymus with low levels detectable in the spleen and lymph node [22]. Analysis of immortalized pre-B cell lines also demonstrated that LEF-1 was expressed in pre-B cells but not mature B cells or plasma cells. During embryogenesis, the fetal site of hematopoiesis is the fetal liver and LEF-1 is expressed strongly in pre-B cells [Reya and Grosschedl, unpublished results]. LEF-1 also has a complex temporal expression pattern in numerous other tissues from E12.5 to E16.5 in embryogenesis, including the mesencephalon, ear, teeth, whisker follicles, pituitary gland, kidney and mammary glands [36, 37].

The LEF/TCF family of HMG transcription factors.

LEF-1 and three other closely related transcription factors, TCF-1, TCF-3 and TCF-4, comprise the LEF/TCF subfamily of HMG transcription factors (figure 1-6) [38]. This group of proteins shares the same HMG domain and thus may be able to act in a functionally redundant fashion. TCF-1 is of particular interest as it contains considerable homology to LEF-1 as well as an overlapping expression pattern during T cell differentiation [36, 39]. TCF-1 was cloned as a T cell-specific gene encoding a CD3ε enhancer-binding protein [40, 41]. LEF-1 and TCF-1 share virtually identical DNA binding properties and both activate the minimal TCRα enhancer, although LEF-1 is approximately 10-fold more efficient in stimulating the TCRα enhancer than TCF-1 [22, 23, 28, 41]. Additional binding sites for LEF-1 and TCF-1 have been identified in transcriptional control regions of several other T lymphocyte-specific genes including

Figure 1-6 The LEF1/TCF subfamily of HMG proteins. LEF-1, TCF-1, TCF-3 and TCF-4 share a practically identical DNA-binding domain (the HMG domain) as well as an N-terminal β-catenin binding domain. LEF-1 differs from the TCF proteins in that it contains a context-dependent activation domain which mediates protein:protein interactions with the transcription factor ALY.


β-catenin binding domain
Context dependent activation domain
HMG domain
56
99
217
287
394
LEF-1
Interaction with ALY
55
299
372
TCF-1
TCF-3
TCF-4

ADA [42, 43], CD4 [44], TCRβ, and TCRδ [45, 46]. Although both *Lef1* and *Tcf1* genes are expressed during T cell differentiation within the thymus and in mature peripheral T cells [39], *Tcf1* transcripts are significantly more abundant than *Lef1* transcripts [28]. TCF-1 deficient mice have been shown to display an incomplete block in thymocyte differentiation, resulting in an accumulation of immature $CD8^+$ single positive cells and a significant reduction of mature T cells in the periphery [39]. In addition, extra-thymic development of intestinal γ/δ- and liver $NK1^+$ T cells is impaired in $Tcf1^{-/-}$ mice [47]. TCF-1-deficient mice, however, have normal expression of the TCRα gene suggesting that TCF-1 does not regulate TCRα transcription *in vivo*.

Like LEF-1, all of the TCF proteins have also been shown to interact through their respective amino termini with β-catenin in response to Wnt/Wingless signaling [48-50]. Expression of TCF-1 and β-catenin together in a T cell line is able to activate transcription of an artificial reporter gene [51], suggesting that Wnt signalling through LEF/TCF family members could play a role in T cell differentiation or function. The investigators also used lithium to inactivate a negative regulator of Wnt signaling, glycogen synthase kinase-3β (GSK-3β), and demonstrated that inhibition of GSK-3β activated TCF-1-dependent transcription in a fibroblast line, but not in the T cell line. This illustrates that Wnt signaling could potentially influence LEF/TCF mediated transcription during T lymphocyte differentiation, however it may act using a mechanism independent of GSK-3β inactivation.

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Chapter 2

Characterization of the LEF-1-deficient mouse

SUMMARY

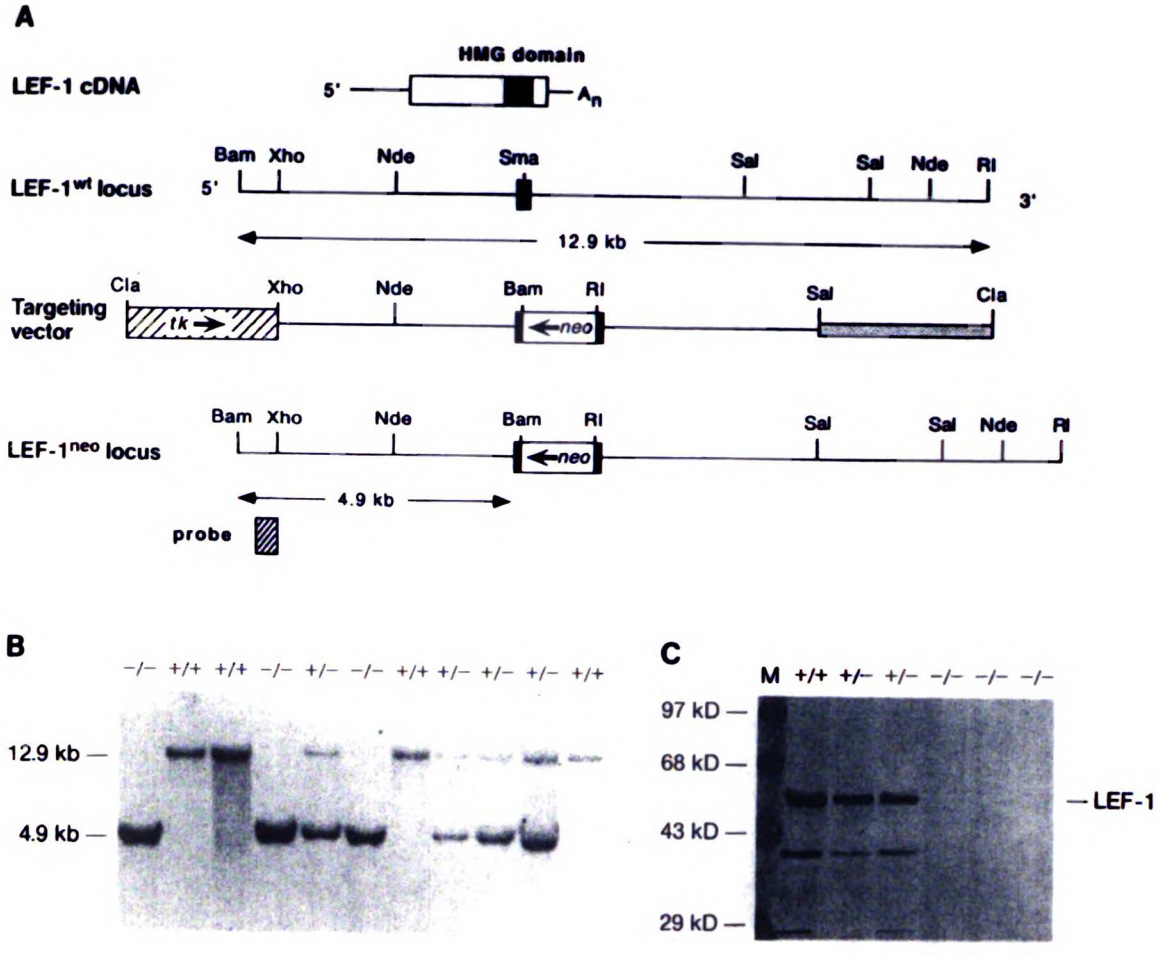
Lymphoid Enhancer Factor-1 (LEF-1) is a member of the HMG family of transcription factors. The expression pattern of LEF-1 in lymphocyte cell lines as well as evidence that LEF-1 regulates TCRα transcription suggested that LEF-1 might be involved in the regulation of lymphocyte differentiation. This chapter details the immunological phenotype of the LEF-1-deficient murine immune system by investigating B and T lymphocyte differentiation and function. These data show that while the loss of expression of functional LEF-1 does affect some of the characteristics of B lymphocyte development, LEF-1 is not essential for either B or T cell differentiation.

RESULTS

Generation of the LEF-1-deficient mouse

In order to study the *in vivo* role of LEF-1 in determining lymphocyte differentiation, Rong-Guo Quo and Courtney van Genderen produced a targeted disruption of the *Lef1* gene in mice [1]. The targeting construct was generated from an 8kb *Lef1* DNA fragment in which the *phosphoglycerokinase (PGK)-neo* gene was inserted into the second exon of the HMG domain of LEF-1 (figure 2-1A). Previous analysis of amino- and carboxy-terminal LEF-1 polypeptides indicated that the HMG domain of LEF-1 is essential for DNA binding [2]. To enrich for homologous recombinations, the construct also included a *PGK-thymidine kinase (TK)* gene in the plasmid [3]. The *Lef-neo-TK* targeting construct was linearized and electroporated into D3 embryonic stem (ES) cells, and clones were doubly selected for the presence of the *neo* gene and the loss of the *TK* gene [4]. Targeted ES clones were injected into C57BL/6 blastocysts and resulting chimeric mice with ES cell contribution exceeding 70% were crossed with C57BL/6 wild-type mice. Multiple chimeras from one clone transmitted the targeted allele through the germ line. Crossing the *Lef1* heterozygous mutant mice, who showed no overt physical defects, generated 74 litters with 23% of the offspring homozygous for the mutation. The genotypes of the offspring were determined by DNA blot analysis of genomic DNA digested with *Bam*HI and *Eco*RI and hybridized with a flanking probe (figure 2-1B). Homozygous mutant (-/-) mice had a drastically impaired viability with only 63% surviving the first week after birth and only 5% surviving the second week. No homozygous mutant survived to weaning, indicating that they carry a recessive mutation in an essential gene.

Figure 2-1 Targeted disruption of the murine *Lef1* locus. (A) The *Lef1* cDNA with the HMG domain shown as a solid box is indicated at the top. The line below represents the wild-type *Lef1* locus in the region of the HMG domain. The targeting vector includes a *PGK-neo*^{r} gene, inserted into the *Sma*I site residing in the 3' exon of the HMG domain, and a *tk* gene. Transcriptional polarity of the *neo*^{r} and *tk* genes are indicated by the arrows. pBR vector sequences are represented by a shaded box. Site for restriction enzymes are indicated above the lines, and the length of the fragments generated by a *Bam*HI-*Eco*RI digest that hybridize with a genomic flanking probe are indicated below. (B) DNA blot analysis of genomic DNA from a representative litter generated by the mating of heterozygous mutant LEF-1-deficient mice. DNA was digested with *Bam*HI-*Eco*RI and probed with the fragment shown in A. The 12.9- and 4.9kb fragments are generated from the wild type and mutant *Lef1* allele, respectively. (C) Immunoblot analysis of nuclear extract from pre-B cells that were obtained from wild type, heterozygous and homozygous mutant LEF-1-deficient mice by transformation with Abelson MuLV. The blot was incubated with purified polyclonal antibodies directed against LEF-1 as described in [5], and LEF-1 was visualized by an alkaline phosphatase secondary antibody. The position of the predominant 55 kD LEF-1 polypeptide is indicated. A minor 39 kD LEF-1 polypeptide is visible, which may be a degradation product or a product of an alternatively spliced *Lef1* transcript. Equivalent amounts of nuclear extract were used in each lane as confirmed by staining a parallel blot with Ponceau S (data not shown).


A
HMG domain
LEF-1 cDNA
5'
An
Bam Xho Nde Sma Sal Sal Nde RI
LEF-1wt locus
5'
3'
12.9 kb
Cla Xho Nde Bam RI Sal Cla
Targeting vector
tk
neo
Bam Xho Nde Bam RI Sal Sal Nde RI
LEF-1neo locus
neo
4.9 kb
probe
B
−/− +/+ +/+ −/− +/− −/− +/+ +/− +/− +/− +/+
12.9 kb
4.9 kb
C
M +/+ +/− +/− −/− −/− −/−
97 kD
68 kD
43 kD
29 kD
LEF-1

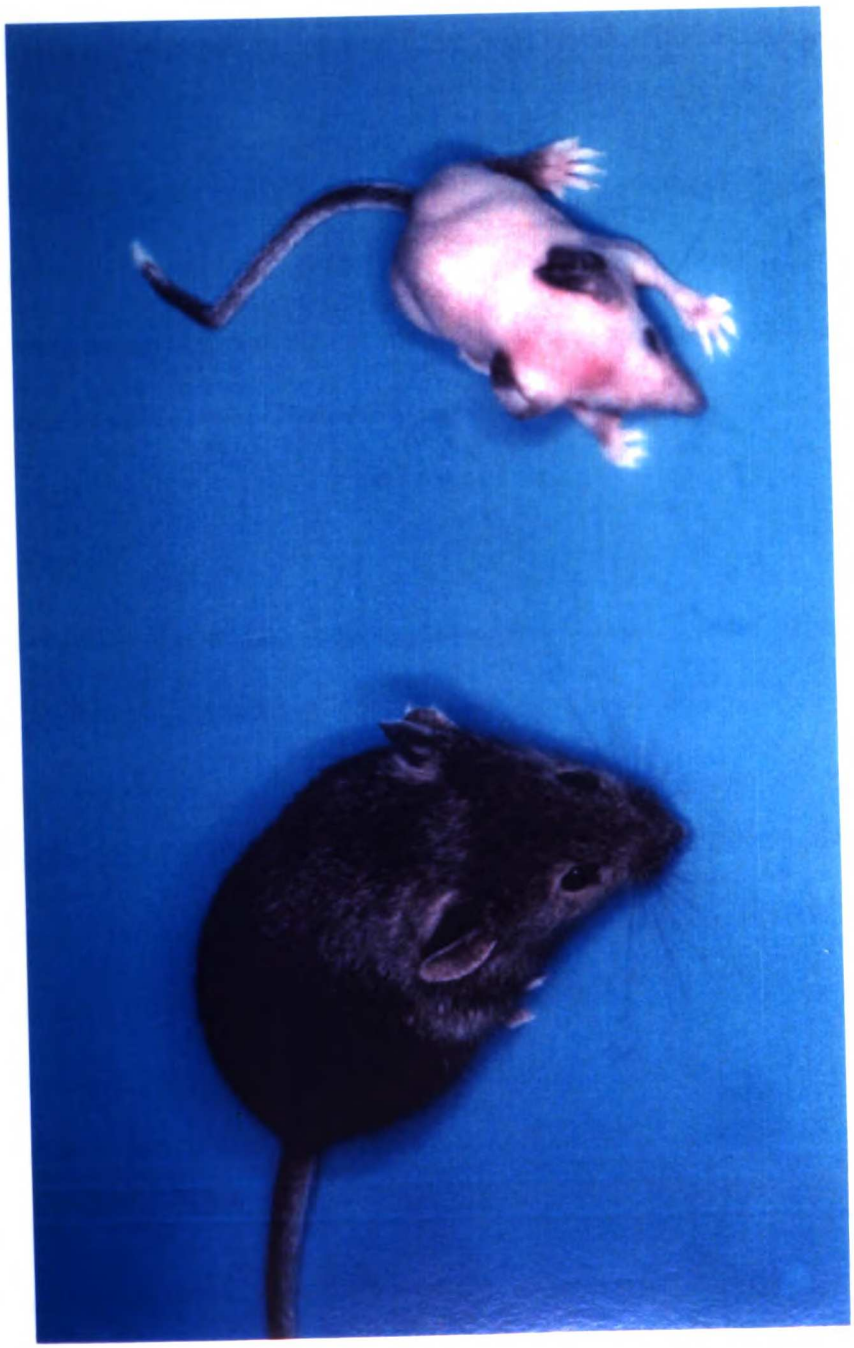
Expression of LEF-1 in mutant mice.

LEF-1 has been previously shown to be expressed in pre-B cell lines [5]. To confirm that the targeted *Lef1* allele does not produce functional LEF-1 protein, we derived pre-B cell lines from the bone marrow of 10-day-old wild type (+/+), heterozygous (+/-), and homozygous mutant mice (-/-) by transformation with Ableson murine leukemia virus (MuLV). We prepared nuclear extracts from the pre-B cell lines and assayed for the presence of LEF-1 protein by immunoblot analysis (figure 2-1C). Incubation of the immobilized protein with antibodies directed against LEF-1 protein allowed for detection of 55- and 39-kD LEF-1 polypeptides in pre-B cells from $Lef1^{+/+}$ and $Lef1^{+/-}$ mice. In contrast, these polypeptides were not detected in pre-B cell extracts from $Lef1^{-/-}$ mice, indicating that the mutation in the *Lef1* gene eliminates its protein expression.

Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice.

The LEF-1-deficient mouse presents a wide range of physiological defects. The most obvious phenotype is the lack of body hair and vibrissae (whiskers) (figure 2-2). In homozygous mutant mice, some rudimentary hair without pigmentation becomes visible at approximately day 9 after birth but this is progressively lost after day 12. Moreover, the mutant mice had a pointed snout and were significantly smaller than sibling heterozygous and wild type mice. In addition, LEF-1-deficient mice lack teeth, mammary glands and the mesencephalic nucleus of the trigeminal nerve [1]. Together,

Figure 2-2 Phenotype of a LEF-1-deficient mouse. The homozygote mutant mouse (right) at day 16 after birth lacks whiskers and body hair. Moreover, the snout has a pointed appearance and the animal is significantly smaller compared to a wild type sibling mouse (left).



the pattern of these defects suggests an essential role for LEF-1 in the formation of several organs and structures that require inductive tissue interactions.

LEF-1-deficient B lymphocytes express abnormal levels of the surface antigens BP-1 and MHC class II.

LEF-1 is expressed in the B-lymphocyte lineage in a temporally restricted expression pattern. All immortalized murine pre-B cell lines express LEF-1 while mature B cell lines and plasma cell lines do not [5]. This pattern of expression is also seen in primary murine lymphocytes. Tannishtha Reya has shown that LEF-1 is expressed in both fetal liver and adult bone marrow in primarily the pro-B cell fractions B and C and not in mature B cells from the spleen and adult bone marrow [Reya and Grosschedl, unpublished results]. These data suggested that LEF-1 would play a role in directing early B cell differentiation and would not be necessary for mature B cell functions.

Analysis of the LEF-1 deficient B lymphocytes was initially performed using antibodies against the B cell antigens B220, CD43, IgD, and IgM (figure 2-3). In the bone marrow and the adult spleen, mature B cells did develop in normal frequencies when compared to wild-type mice demonstrating that B cells could mature in the absence of LEF-1. However, closer analysis of the early stages of differentiation revealed that LEF-1-deficient B cells have a decrease in expression of the surface antigen BP-1 [Reya and Grosschedl, unpublished results] and an increase in the absolute surface expression of MHC class II (figure 2-4). While these changes in surface antigen expression are striking, a clear block in differentiation was not seen as more mature B cell populations are detectable (figure 2-3). To further address the question of whether or not B cell

Figure 2-3 B cell differentiation in LEF-1-deficient mice. Two-color flow cytometry analysis of LEF-1-deficient B cells using antibody against mouse B220, CD43, IgD and IgM. The left panels represent neonatal bone marrow cells and the right panels are neonatal splenic B cells. The genotypes of the B cells are indicated along the top row.

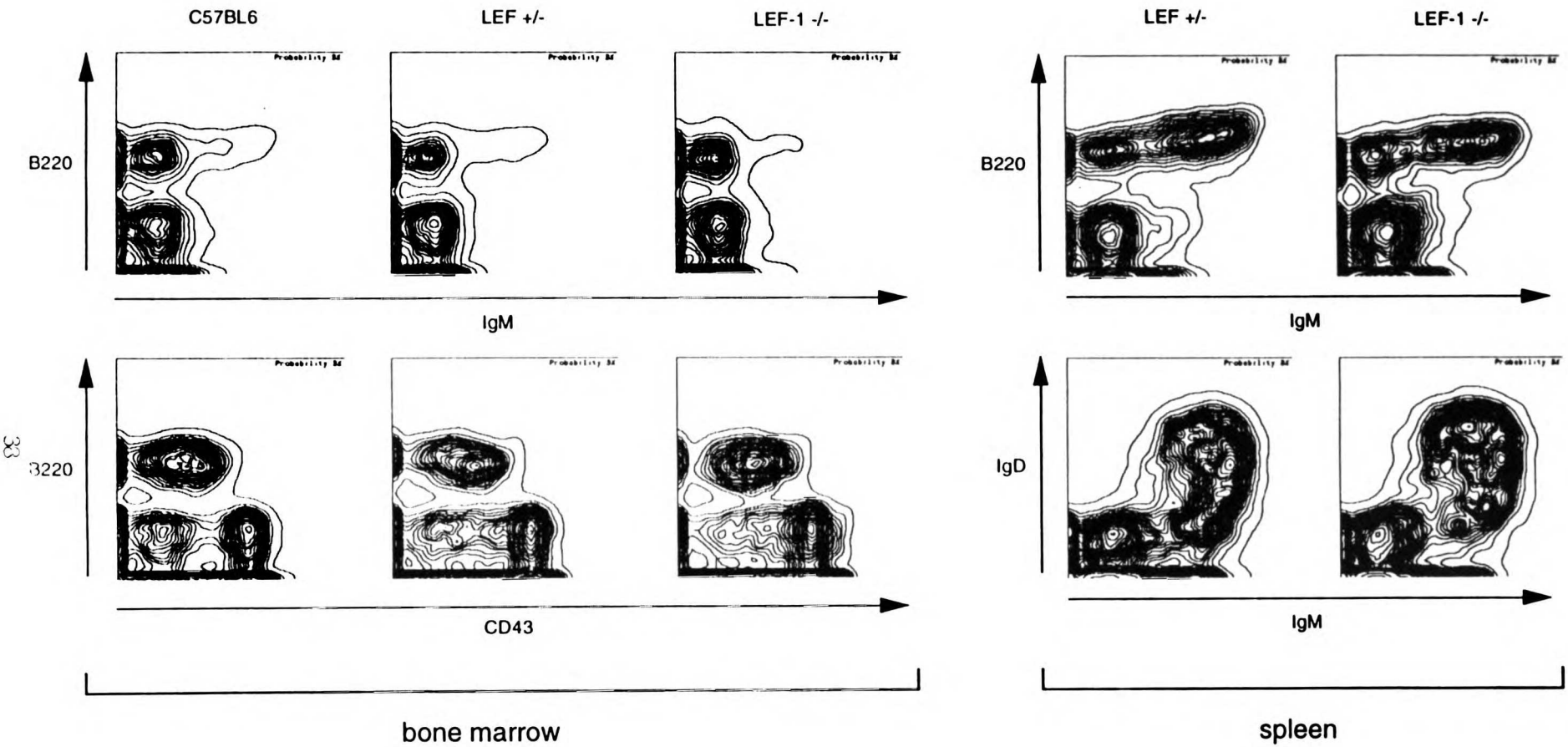
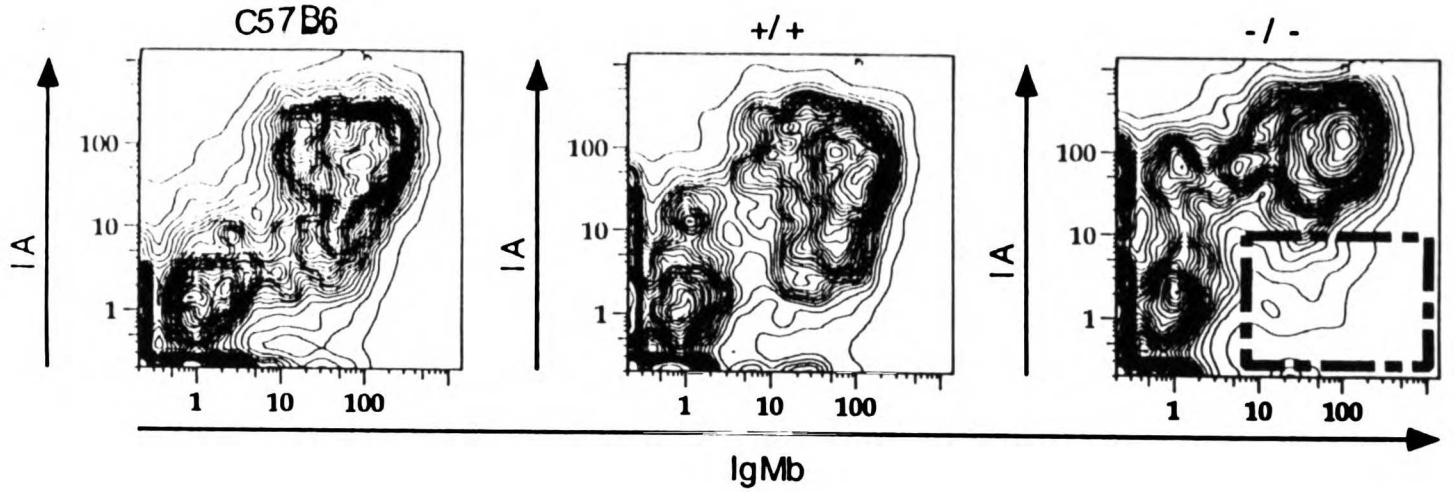
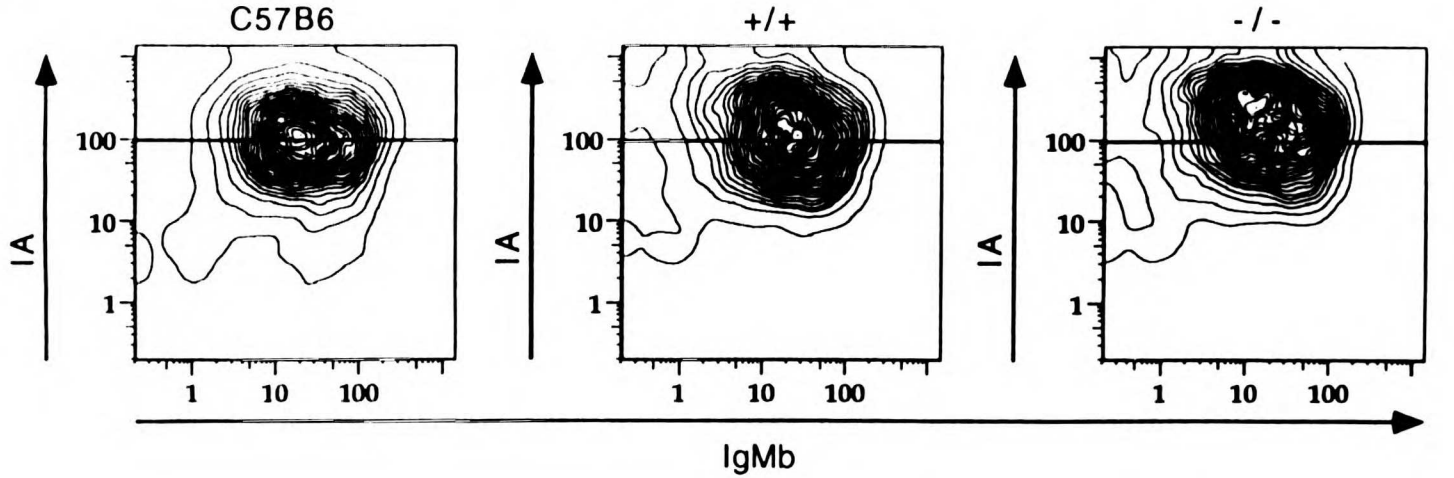

C57BL6
LEF +/-
LEF-1 -/-
B220
IgM
3220
CD43
bone marrow
LEF +/-
LEF-1 -/-
B220
IgM
IgD
IgM
spleen

Figure 2-4 Expression MHC class II is abnormal in LEF-1-deficient B cells. (A) Three-color flow cytometry analysis of LEF-1-deficient B cells using antibody against mouse B220, IA and IgM. All cells are $B220^+$ (gated) and are derived from neonatal bone marrow. (B) Three-color flow cytometry analysis of LEF-1-deficient B cells using antibody against mouse B220, IA and IgM. All cells are $B220^+$ (gated) and are derived from either adult C57BL/6 mice or adoptive transfer experiments.

A. B220+ Bone Marrow, neonate



B. B220+ Spleen, adoptive transfer



differentiation is affected by the loss of functional LEF-1, we analyzed the rearrangement of the immunoglobulin heavy chain in LEF-1-deficient B lymphocytes.

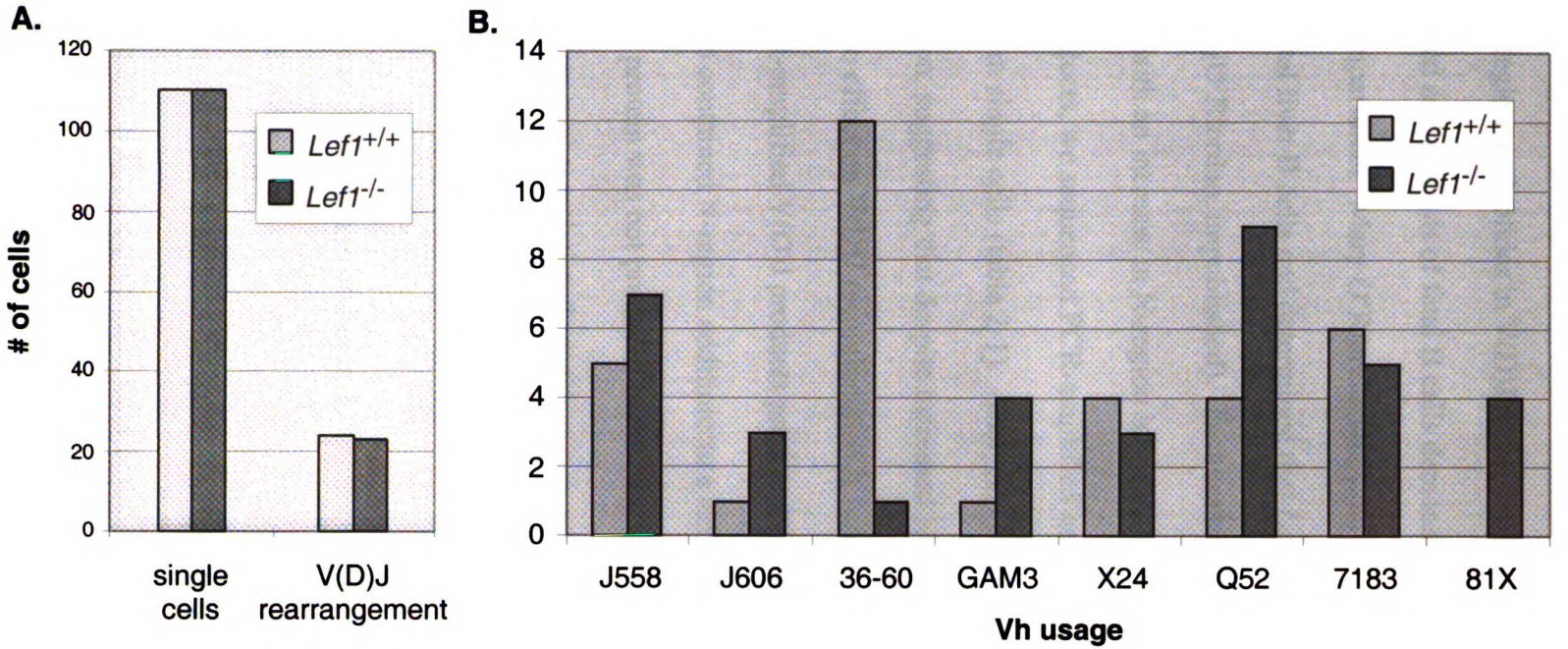
Immunoglobulin heavy chain V(D)J rearrangement occurs normally in LEF-1-deficient animals.

The generation of a productive immunoglobulin receptor is one of the primary goals of B cell differentiation and observing the degree to which this essential process occurs is a good measure of the capability of B cells to differentiate. To assess whether *Lef1*$^{-/-}$ mice are able to complete this critical process, we isolated B220^{+} cells and determined the frequency and usage of immunoglobulin heavy chain V(D)J rearrangements. The assay we used involved sorting single B cells from neonatal fetal liver. The cells were sorted directly into a lysis buffer and the genomic DNA of each sample was amplified using a series of nested PCR amplifications [6, 7]. The results of the assay show that both the absolute recombination frequency (figure 2-5A) and the immunoglobulin heavy chain V_H usage (figure 2-5B) was comparable between the wild type and *Lef1*$^{-/-}$ fetal liver B cells. This demonstrates that LEF-1-deficient B lymphocytes are able to differentiate into mature B cells with no block in development and with no alteration to the immunoglobulin heavy chain repertoire produced.

LEF-1-deficient fetal liver B cells have an elevated level of expression of TdT, but do not exhibit a concurrent increase in immunoglobulin heavy chain N-region addition.

Since the LEF-1 is expressed highly in fetal liver pro-B cells we were interested in determining if LEF-1 was responsible for the regulation of fetal-specific B cell

Figure 2-5 Immunoglobulin heavy chain V(D)J rearrangement in *Lef1*$^{-/-}$ B cells. 110 single B220^{+} cells from *Lef1*$^{+/+}$ and *Lef1*$^{-/-}$ neonatal fetal liver (day 0) were sorted individually using a FACStar Plus. Genomic DNA from each single cell was subjected to a two-step PCR amplification to generate V(D)J rearrangement products as described [6]. (A) Summary of single-cell PCR analysis showing the number of total single cells analyzed for each genotype and the number of single cells yielding a V(D)J rearrangement. (B) Relative Vh usage of eight Vh families as defined by single-cell PCR analysis.


A.
120
100
80
60
40
20
0
# of cells
Lef1+/+
Lef1-/-
single cells
V(D)J rearrangement
B.
14
12
10
8
6
4
2
0
Lef1+/+
Lef1-/-
J558
J606
36-60
GAM3
X24
Q52
7183
81X
Vh usage

characteristics. One such phenotype is the expression of the enzyme terminal deoxytransferase (TdT). TdT is found in abundance in bone marrow B cells and causes the insertion of N-region nucleotides in V(D)J rearragement. In fetal B cells however, TdT is not expressed and analysis of fetal B cells demonstrates that there is no N-region addition in this compartment. Mary O'Riordan analyzed the mRNA levels of TdT in LEF-1-deficient fetal liver B cells and determined that *Lef1*$^{-/-}$ B cells upregulate the expression of TdT (O'Riordan, unpublished). To determine if this increase in TdT mRNA correlated with an increase in N-region insertion in immunoglobulin rearrangement products, we sequenced PCR-amplified immunoglobulin heavy chain recombinations from single cells (table 2-1). Analysis of the fetal liver B cells revealed no N-region addition, suggesting that despite increased levels of TdT mRNA in LEF-1-deficient B cells, no effect on V(D)J recombination is induced. As a control, we also sequenced the PCR-amplified V(D)J products produced from splenic B cells and found that both genotypes contained N-region additions to a similar degree. This would suggest that either the TdT protein was not produced in the fetal liver LEF-1-deficient B cells or that other activities or post-translational modifications are required to initiate N-region addition.

CD5$^+$ B1 cells require LEF-1 expression in 129/SvJ/C57BL/6 mice, but not in backcrossed C57BL/6 animals.

Another characteristic thought to be specific to fetal liver B cells [8] is the ability to produce a population of B cells present in mice that is considered distinct from the conventional B cells. The CD5$^+$ B1 cells (B1a cells) (reviewed in [9]) in the adult mouse

Table 2-1. Immunoglobulin heavy chain rearrangement in LEF1-deficient B cells

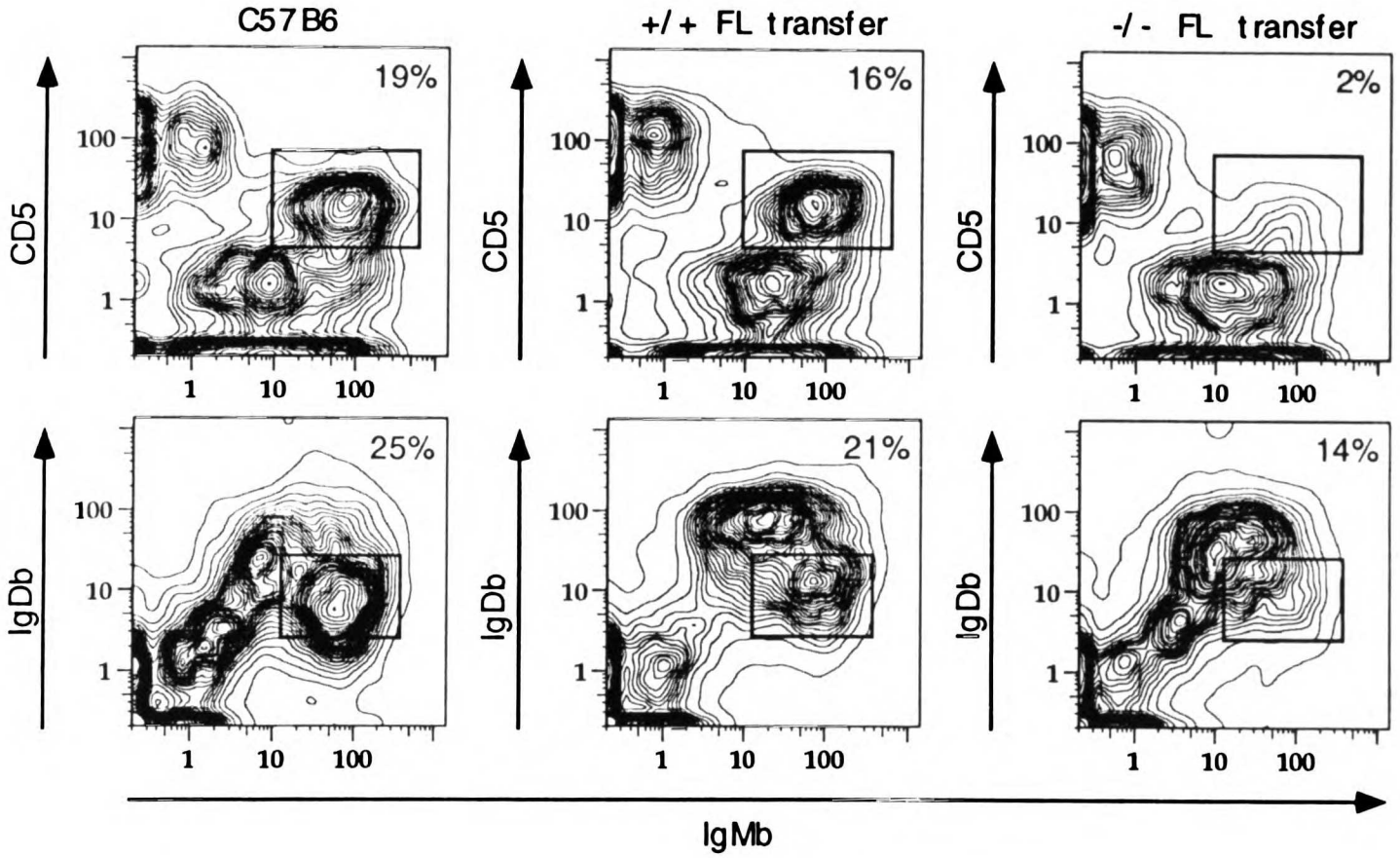
Clone	Dh	N	Jh	Dh	Jh	RF
Neonate (p0), fetal liver						
Lef1-/-						
123-1	T CTATGA TGG TTA CTA		G TTT GCT TAC TGG	Dsp2.9	J3	3
123-5	TCT ATG ATG GTT AC		(G G)CC TGG TTT GCT TAC TGG	Dsp2.9	J3	2
123-10	TC TAC TAT GAT TAC GAC		(G)CC TGG TTT GCT TAC TGG	Dsp2.2	J3	1
124-2	TT TAT TAC TAC GGT AGT AGC TAC		TAT GCT ATG AAC TAC TGG	Dfl16.1	J4	1
Lef1+/+						
9103-1	TCT ATG ATG GTT AC		(G G)CC TGG TTT GCT TAC TGG	Dsp2.9	J3	2
9103-5	C CTA CTA TAG TAA C		CT TAC	Dsp2.x	J3	3
9104-3	TT TAT TAC TAC GGT AG		TGGCT ATG GAC TAC TGG	Dfl16.1	J4	1
9104-4	TC TAC TAT GAT TA		T TAC TAT GCT ATG GAC TAC TGG	Dsp2.2	J4	1
9104-5	CC TAC TAT AGT AAC		TAC TAT GCT ATG GAC TAC TGG	Dsp2.x	J4	1
9104-6	TC TAC TAT GGT AAC		GCT ATG GAC TAC	Dsp2.1/.5	J4	1
9104-11	TTT ATT ACT ACG GTA GTA	TGC T	TG GAC TAC TGGTGG	Dfl16.1	J4	2
adoptive transfer, spleen						
Lef1-/-						
KO3	T TTA TTA CTA CGG NNG NAG CTA	A	AT TAC TAT GCT ATG GAC TAC TGG	Dfl16.1	J4	3
KO5	TC TAC TAT GAT TAC G	G	C TAT GCT ATG GAC TAC TGG	Dsp2.2	J4	1
KO8	TC TAC TAT GGT AAC TAC	GAC	AT GCT ATG GAC TNC TGG	Dsp2.1/.5	J4	1
KO11	TT TAT TAC TAC GG	G ACG GGG	GCT ATG GAC TAC TGG	Dfl16.1	J4	1
KO12	TC TAC TAT GAT TAC GAC	TAC GGT NTN	TAC TAT GNT ATG GAC TAC TGN	Dsp2.2	J4	1
Lef1+/+						
WT3	TC TAC TAT GGT AAC TAC	GAC	AT GCT ATG GAC TAC TGG	Dsp2.1/.5	J4	1
WT9	CC TAN TAN TNC AN	G TAN AAN	AT GCT ATG GAC TAC TGG	Dsp2.x	J4	1
WT11	TC TAC TAT GAT TAC GAC	GTG	GCT ATG GAC TAC TGG	Dsp2.2	J4	1
WT12	TCT ACT ATG ATT A	NN GGN	NTN GNC TAC TGN	Dsp2.?	J4	2
WT14	TT TAT TAC TAC GGT	CT	C TAT GCT ATG GAC TAC TGG	Dfl16.1	J4	1
WT15	TC TAC TAT GAT T	CC CTC TAT C	CT ATG GAC TAC TGG	Dsp2.2	J4	1

V(D)J immunoglobulin heavy chain products were produced using single-cell PCR as originally described in [6] and modified in [7]. Rearrangements were gel isolated,cloned into pBluescript and sequenced. Identification of specific Dh regions was performed as described [6].

are defined as a self-replenishing population in the peritoneal cavity where they represent a large percentage of the B cells present [10, 11]. They can be distinguished from conventional B cells by distinct levels of expression of the surface antigens B220, HSA, IgD and Mac-1. A $CD5^-$ B1 (B1b) cell population also exists and has been shown to share many of the characteristics and properties of the B1a population [12-14]. B1 cells are thought to be responsible for generating the immune response against T cell-independent (TI) antigens [14] and mice lacking the B1 populations are unable to respond to TI antigenic challenges [15, 16]. In addition, B1a cells are thought to produce most of the serum IgM [15] and most of the B cell-produced IL10 [17].

To determine if LEF-1 is required for the production of the $CD5^+$ B1 cell population, we performed adoptive transfer experiments using *Lef1*$^{-/-}$ fetal liver precursors [18]. This was necessary, as LEF-1-deficient mice do not survive to an age where these experiments could be performed. The recipient mice were immunodeficient BALB/C SCID mice, each of whom received by I.V. transfer $1x10^7$ cells fetal liver cells. Eight weeks after the adoptive transfer, each mouse was screened for the presence of mature donor B and T cells in the peripheral blood by flow cytometry. We next analyzed *Lef1*$^{-/-}$ peritoneal cavity cells from the successful adoptive transfer experiments for the presence of B1a cells. LEF-1-deficient adoptive transfers were initially performed with fetal tissue from animals of a mixed background (D3:C57BL/6) and analysis of peritoneal cavity lymphocytes revealed a lack of $CD5^+$ B1 cells (figure 2-6). The loss of the population was not due to an inability to produce the CD5 antigen as LEF-1-deficient T cells express normal levels of CD5 ($CD5^+/IgM^-$ cells; figure 2-6). Additionally, the loss of the population was verified by a reduced number of IgD^{low}/IgM^+ cells in the peritoneal

Figure 2-6 $CD5^+$ B1 cells require LEF-1 expression. Two-color flow cytometry analysis of peritoneal cavity cells from adoptive transfer experiments using antibodies against murine CD5, IgD and IgM. Genotypes are indicated above the top row.


C57B6
+/+ FL transfer
-/- FL transfer
19%
16%
2%
25%
21%
14%
CD5
IgDb
IgMb
100
10
1
1
10
100

cavity (figure 2-6). These results were true for all mixed background LEF-1-deficient adoptive transfers tested (table 2-2). After backcrossing the mice fifteen generations on the C57BL/6 background, we repeated the adoptive transfers in SCID mice and assayed for the ability of the donor cells to produce B1a cells by flow cytometry analysis (figure 2-7). The $CD5^+$ B1 cell population in this group of adoptive transfers was unaffected by the loss of LEF-1 protein and appears to be present in equivalent amounts in each genotype tested. This result suggests that the early data was the result of the mixed lineage of the donor tissue as no other variable was changed in the protocol used. There are many possible explanations for how this would occur but we cannot conclude from our data the exact mechanism by which this strain difference appears. Therefore, while LEF-1 may be essential for $CD5^+$ B1 cell differentiation, this requirement is present in a strain-specific manner.

***Lef1*$^{-/-}$ B lymphocytes are able to proliferate in response to anti-IgM stimulation.**

While differentiation of B lymphocytes appears to proceed unimpeded in LEF-1-deficient mice, it is not clear whether the B cell repertoire produced is immunologically functional. When a B cell is stimulated through the immunoglobulin receptor, the mature B lymphocyte is induced to proliferate and differentiate to become a plasma cell. The plasma cells then produce serum antibody that is directed against the specific antigen challenge received.

To address the issue of whether the LEF-1-deficient B cells could proliferate in response to stimulation of the immunoglobulin receptor, we sacrificed several SCID adoptive transfers which had received donor fetal liver from wild type and *Lef1*$^{-/-}$ E16

Table 2-2. Generation of B1a cells in adoptive transfer experiments

Donor genotype	No. of mice	background	CD5+ B1 cells
Lef1$^{+/+}$	18	D3:C57BL/6	18
Lef1$^{-/-}$	36	D3:C57BL/6	0
Lef1$^{+/+}$	10	C57BL/6	10
Lef1$^{-/-}$	10	C57BL/6	10

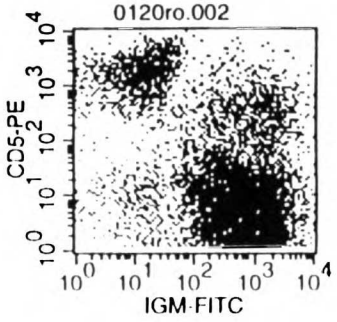
All analysis was performed 10 weeks after intravenous transfer of $1x10^7$ E16.5 fetal liver cells into BALB/C SCID mice. The presence of B1a cells was detected by fluorescence-activated cell sorting, using the antibodies CD5, IgD, IgM, and Mac-1. Only adoptive transfers that were successfully reconstituted with conventional B and T cells are considered in this table.

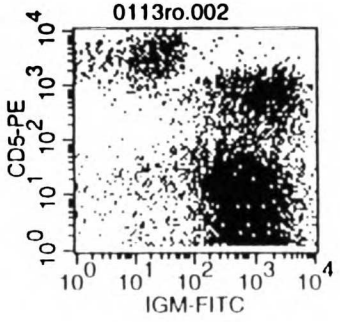
Figure 2-7 B1a cell requirement for LEF-1 is not present in C57BL/6 mice. Two-color flow cytometry analysis of peritoneal cavity cells from C57BL/6 mice and adoptive transfer experiments using antibodies against murine CD5 and IgM. Genotypes are listed on each row.

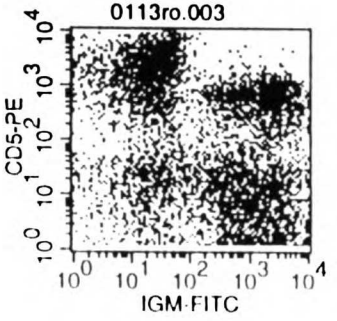
Adoptive transfers,
10 wks

C57BL/6,
10wks old

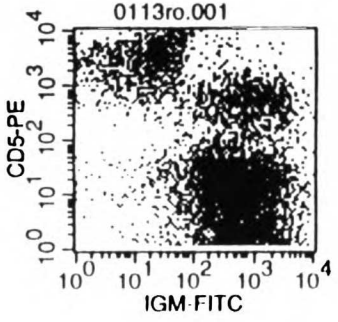
Lef1+/+

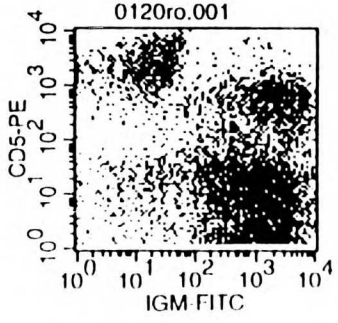






Lef1-/-





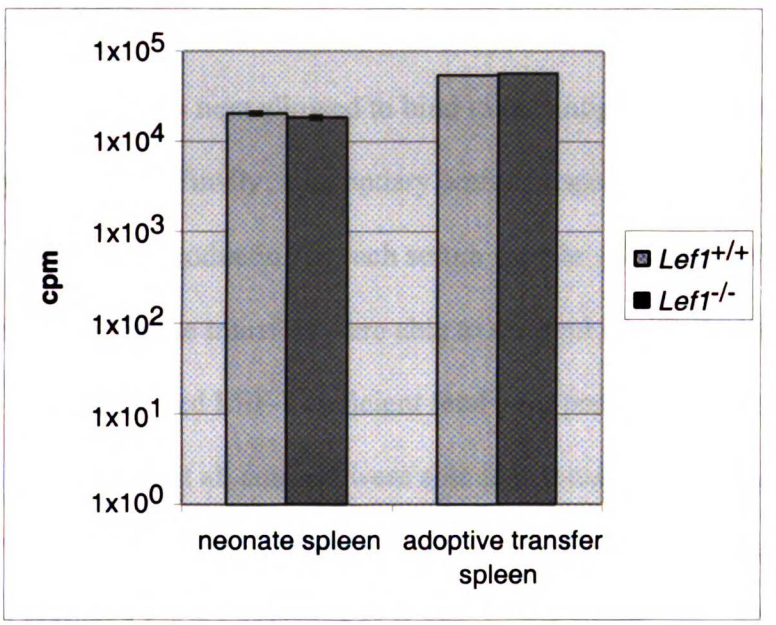
embryos. Splenic B cells from these animals were then stimulated in culture with 10μg/ml of anti-IgM antibody for 48 hours and the proliferation was determined by the incorporation of a 12 hour pulse of 1μCi of 3H-thymidine. The results were collected by liquid scintillation spectrophotometry and showed that both genotypes were able to proliferate in response to the stimulation (figure 2-8). This result illustrates that LEF-1 deficient B cells can proliferate in response to immunoglobulin receptor stimulation.

LEF-1-deficient B cells can respond to both T-independent and T-dependent antigenic challenge.

We next tested the ability of the SCID adoptive transfers to produce a specific antibody response to antigenic challenge. Antigens in general have been subdivided into two main groups, T-independent antigens and T-dependent antigens which denotes whether or not a T cell interaction is necessary for the production of an immune response. Classically, T-dependent antigens have been shown to be unable to generate a response in naturally athymic nude mice and T-independent antigens are able to cause an immune reaction in those same animals. T-dependent antigens, in general, are complex protein antigens and T-independent antigens usually contain repeating determinants that are thought to act by crosslinking B cell antigen receptors.

The SCID adoptive transfers received an injection of PBS and alum, with or without antigen. The antigens that were used in these experiments were TNP-BSA, a T-dependent antigen, and a NIP-KLH, a T-independent antigen. Serum was collected from the animals before the first injection (day 0) and at each time point where the animal received a booster reimmunization (days 14, 21, 28, and 35). As a control, all day 0

Figure 2-8 LEF-1-deficient B cells are able to proliferate in response to anti-IgM stimulation. Triplicate cultures of $1x10^6$ splenic B cells from neonatal mice and adoptive transfer experiments were stimulated with 10μg/ml of an anti-IgM antibody for 48 hours in culture at 37°C. The cells were pulsed with 1μCi of 3H-thymidine and incubated 12 hours before harvesting. Radioactivity incorporation was measured by liquid scintillation spectrophotometry.


1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
cpm
neonate spleen
adoptive transfer spleen
Lef1+/+
Lef1-/-

samples were checked by ELISA for the presence of serum immunoglobulin to rule out a general problem in B cell function (figure 2-9). The result of this assay showed that LEF-1-deficient samples contained equivalent resting levels of serum immunoglobulin when compared with wild-type samples, demonstrating that LEF-1 is not required for the production of serum immunoglobulin. The serum samples were then checked for an antigen-specific immune response using another ELISA assay specific for the antigen used. For this assay, the antigen tested was first bound to the well of a 96-well plate. 3µl of serum, in triplicate, was next allowed to bind to the antigen and unbound antibody was washed away with PBS. Finally, a secondary antibody specific for IgG_1 was used to detect specific antibody production in each serum sample (figure 2-10). In most cases (16/20), wild-type adoptive transfers were able to respond to either antigen (table 2-3). The animals which received LEF-1-deficient fetal liver precursors also responded to an equal degree, however, not all animals were able to respond to the antigenic challenge (table 2-3). This unresponsiveness was not due to an incomplete reconstitution as all animals were screened for the presence of B and T cells before use. While it is possible that the *Lef1*$^{-/-}$ B cells have some reduced ability to respond to the challenge, perhaps requiring a higher dosage of antigenic challenge, it is clear that they can be immunocompetent in the absence of functional LEF-1 protein. Taken together, these results show that conventional LEF-1 deficient B cells are able to function in response to either a T-dependent or T-independent antigenic challenge.

Figure 2-9 Resting levels of serum immunoglobulin in adoptive transfers. Two-step serum ELISA analysis of immunoglobulin sampled from unstimulated, pathogen-free adoptive transfer experiments. Donor genotypes are indicated in the legend. Standard deviation represents triplicate samples used.

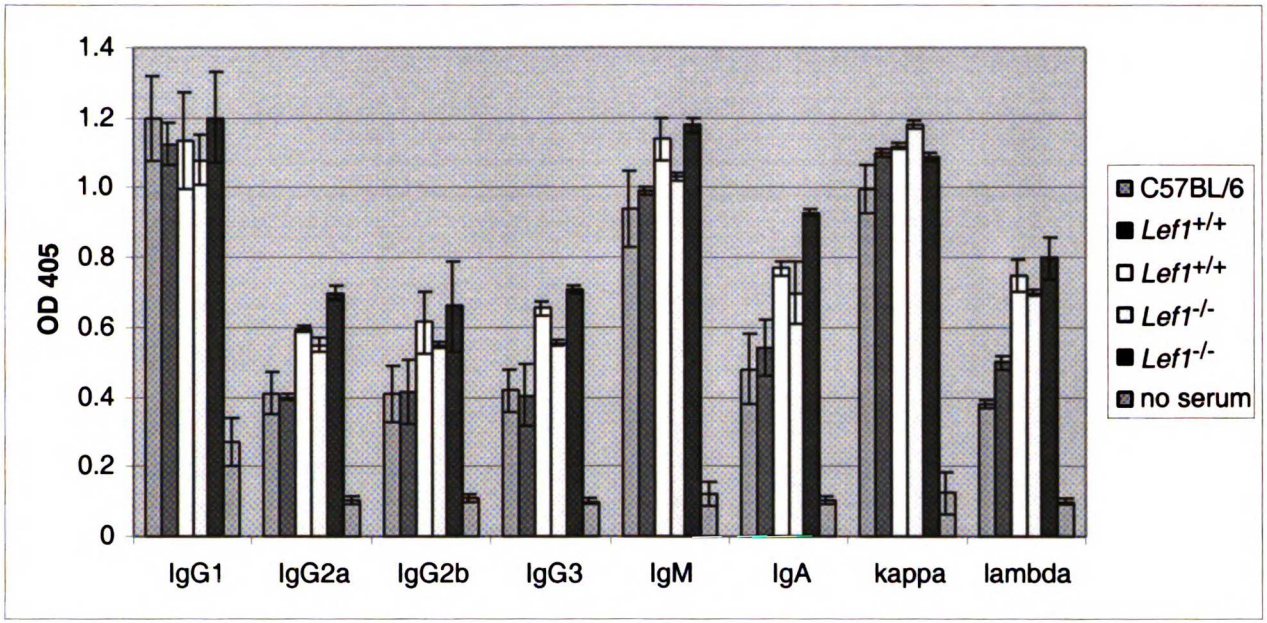

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD 405
IgG1
IgG2a
IgG2b
IgG3
IgM
IgA
kappa
lambda
C57BL/6
Lef1+/+
Lef1+/+
Lef1-/-
Lef1-/-
no serum

Figure 2-10 Specific antibody response to antigenic challenge is unimpaired in LEF-1-deficient mice. Two-step serum ELISA analysis of immunoglobulin sampled from antigen-challenged adoptive transfer experiments. Donor genotypes and the antigen used are indicated along the bottom of the chart. Standard deviation represents triplicate samples used.

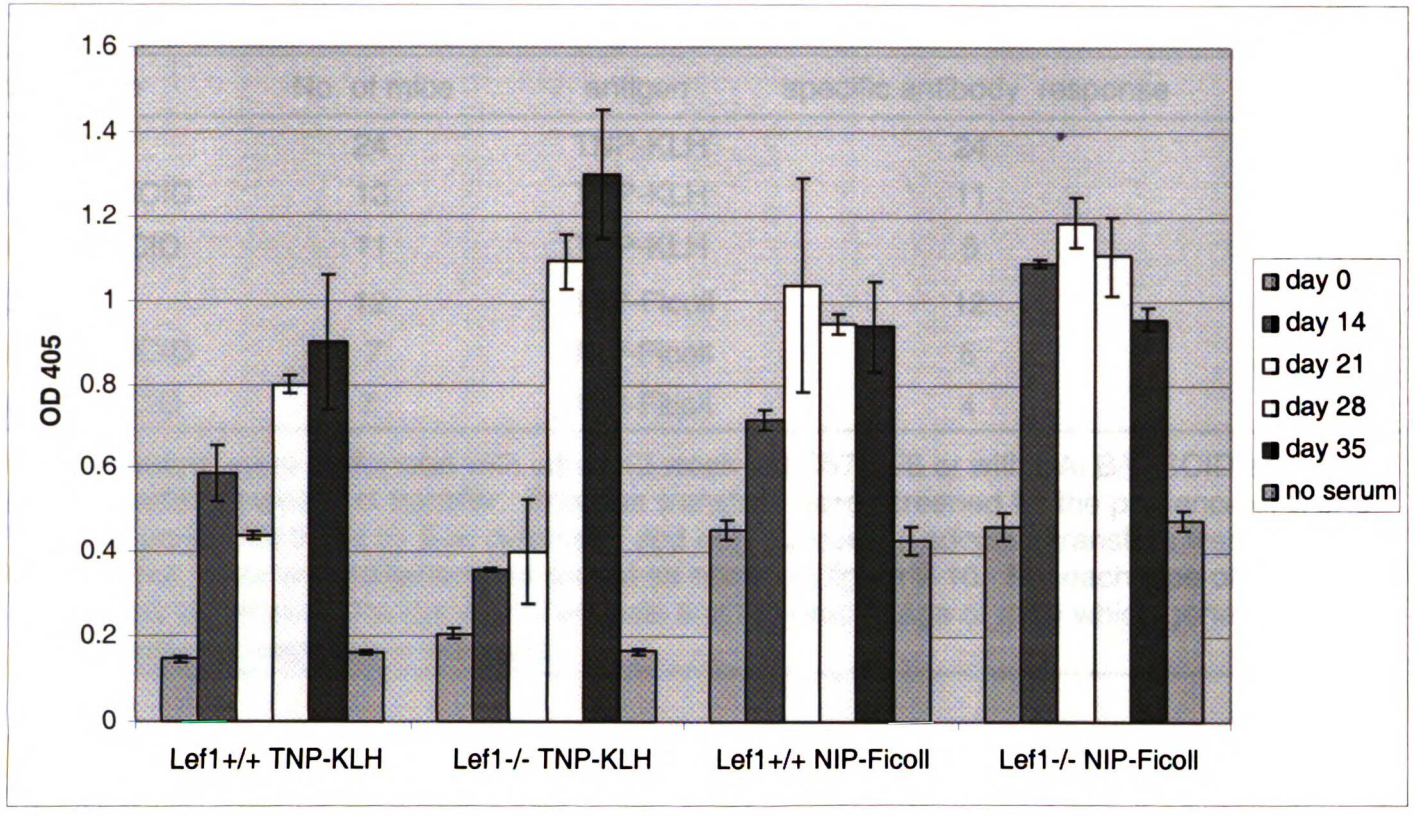

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 405
day 0
day 14
day 21
day 28
day 35
no serum
Lef1+/+ TNP-KLH
Lef1-/- TNP-KLH
Lef1+/+ NIP-Ficoll
Lef1-/- NIP-Ficoll

Table 2-3. Antigenic challenge in C57BL/6 and SCID adoptive transfers

Genotype	No. of mice	antigen	specific antibody response	% of total
C57BL/6	24	TNP-KLH	24	100%
Lef1 $^{+/+}$ SCID	13	TNP-KLH	11	85%
Lef1 $^{-/-}$ SCID	11	TNP-KLH	5	45%
C57BL/6	12	NIP-Ficoll	12	100%
Lef1 $^{+/+}$ SCID	7	NIP-Ficoll	5	71%
Lef1 $^{-/-}$ SCID	7	NIP-Ficoll	4	57%

All experiments were performed with either 12 week old C57BL/6 or with BALB/C SCID adoptive transfers after 10 weeks of transfer. Adoptive transfers were screened for the presence of B and T cells in peripheral blood by flow cytometry and only successful adoptive transfers were used for this protocol. Data was collected and scored as shown in figure 2-10. For each type of mouse used in this experiment, the number of animals and the percentage of mice which generated a specific antibody response is indicated.

LEF-1-deficient T lymphocytes are able to differentiate normally.

LEF-1 is also expressed in primary T cells and T cell lines [5, 19]. In addition, the only well characterized lymphocyte-specific target of LEF-1 transcriptional regulation is the TCRα enhancer [2, 20, 21]. These data suggest that LEF-1 may be important for T lymphocyte differentiation and that the absence of functional LEF-1 protein may lead to a differentiation block similar to the one displayed by mice deficient for the TCRα gene [22]. LEF-1-deficient animals are able to develop a thymus and, while it is reduced in size compared to sibling thymi, the thymus remains approximately proportional with its smaller body size.

An examination of thymocytes from *Lef1* $^{-/-}$ mice by flow cytometry was performed using antibodies against the surface antigens CD4 and CD8, and TCRβ and TCRγ/δ (figure 2-11). No gross defect was observed in the probability plots when comparing the wild type T cells to the thymocytes derived from mice either heterozygous or homozygous for the mutation in the *Lef1* gene. Both the existence of mature single positive CD4 and CD8 cells and the normal surface expression of TCRβ suggested that there was no defect in the production of TCRα in LEF-1-deficient mice. To address this issue more directly, RNA analysis for TCRα using S1 nuclease protection was performed using *Lef1*$^{-/-}$ thymocytes (figure 2-12). Additionally, the cells were analyzed for the expression of the related HMG family member TCF-1. Overall, there was no detectable difference in the amount of either TCF-1 or TCRα RNA between the different genotypes analyzed. This result demonstrates that in the absence of functional LEF-1, TCRα transcription and translation are unaffected. While this analysis suggested that T cell

Figure 2-11 Normal T cell development in LEF-1-deficient mice. Two-color flow **cyto**metry analysis of neonatal thymic T lymphocytes using antibodies against mouse **CD4**, CD8, TCRβ and TCRγ/δ. The genotypes of T cells are indicated along the top row.

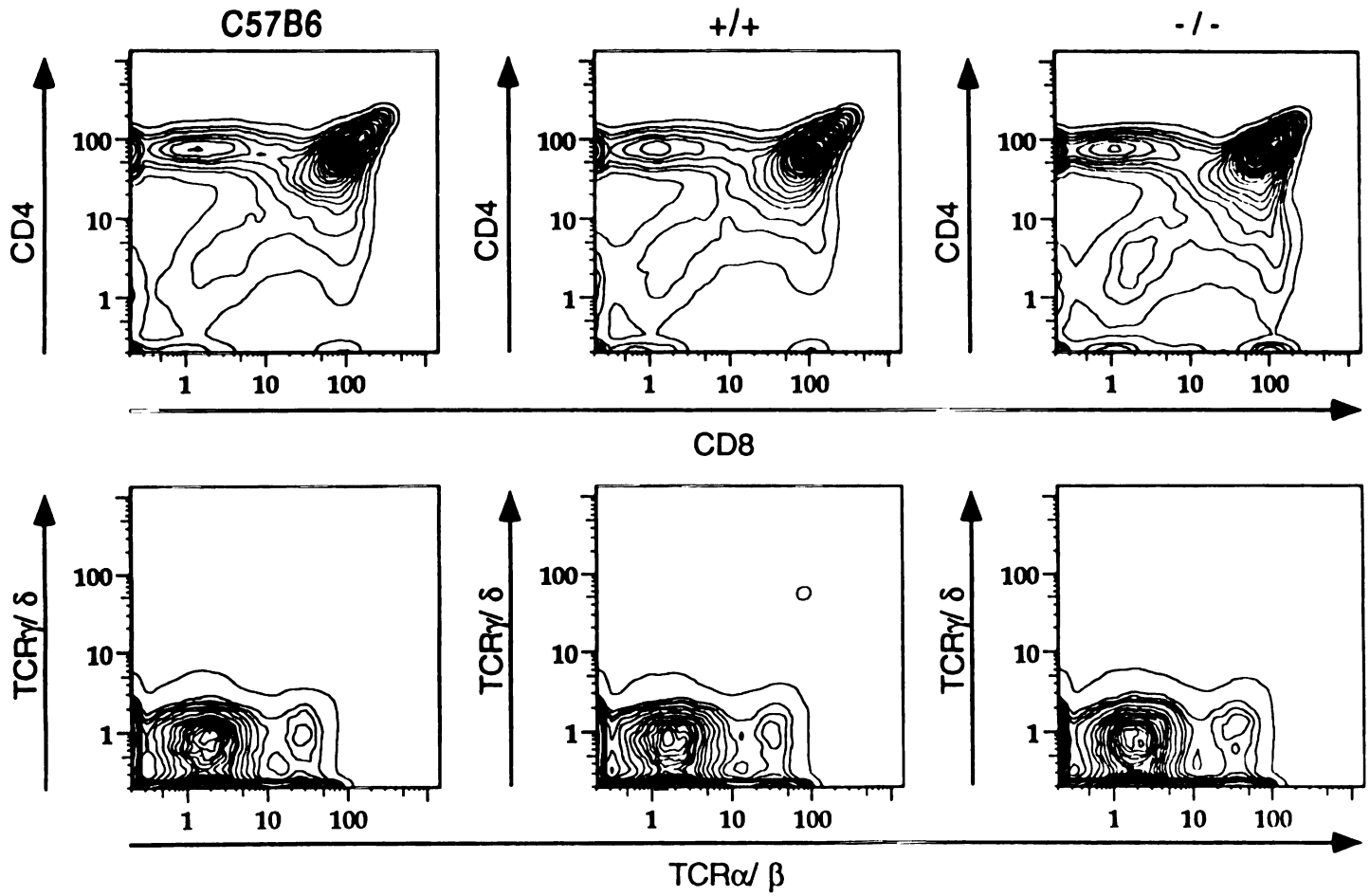
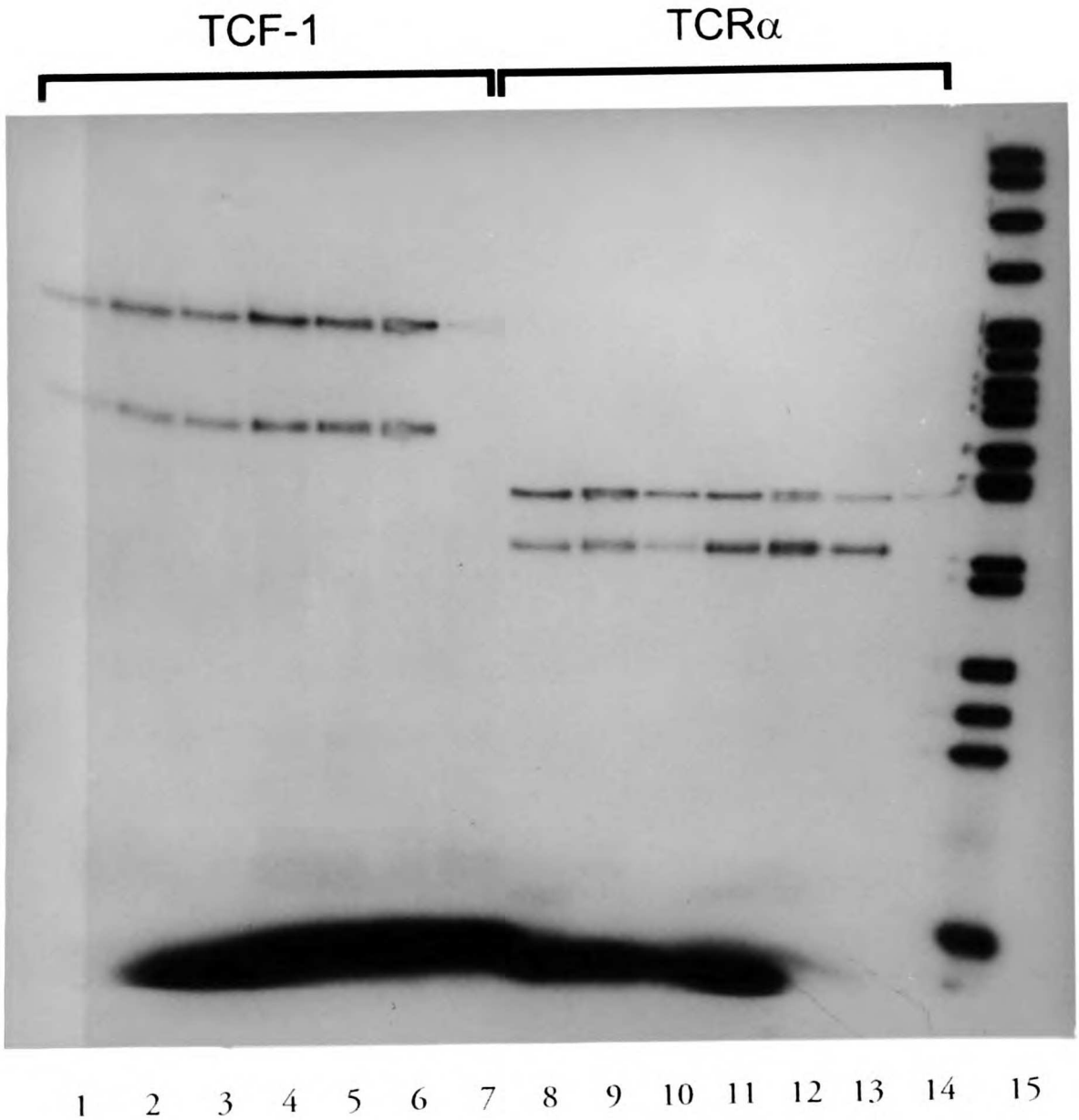

C57B6
+/+
-/-
CD4
CD8
TCRγ/δ
TCRα/β
100
10
1
1
10
100

Figure 2-12 TCRα and TCF-1 expression are unaffected by the absence of LEF-1. S1 nuclease protection assay of thymic T cell mRNA using probes for TCF-1 (lanes 1-7) and TCRα (lanes 8-14). Lanes 1, 4, 8, and 11 are *Lef1*$^{-/-}$. Lanes 2, 3, 5, 6, 9, 10, 12, and 13 are *Lef1*$^{+/+}$. Lanes 7 and 14 contain tRNA.


TCF-1
TCRα
380bp
205bp
143bp
120bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

differentiation proceeded normally, it remained possible that the mature LEF-1-deficient T cells selected may be functionally incompetent to provide an immune response.

Expression of LEF-1 is not required for calcium flux in reponse to TCR engagement.

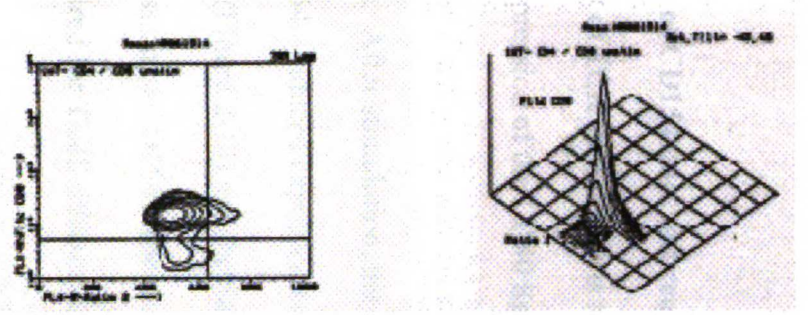
When T cells are stimulated by the binding of ligand to the TCR complex, a series of biochemical processes are initiated. Immediate responses include tyrosine phosphorylation of intracellular proteins and the induction of a calcium flux. These signals trigger effector functions within the T cell such as proliferation, secretion of cytokines and differentiation [23]. To explore whether or not the absence of LEF-1 during thymocyte differentiation affects the ability of the mature T cells to function normally, we isolated T cells from LEF-1-deficient animals. We began testing the initial response to TCR signaling by studying the ability of LEF-1 deficient T cells to produce a calcium flux in response to treatment with either an application of the anti-CD3ε antibody 2C11, which binds to the TCR complex, or, as a control, the addition of the calcium ionophore ionomycin. T cells were first loaded with 1μM of the calcium-binding dye Indo-1-AM at 37°C for 30 minutes. The cells were then washed with PBS and labeled with antibodies against mouse CD4 and CD8. After staining, the cells were stimulated with 10μg of 2C11 and the calcium release was monitored by flow cytometry (Figure 2-13). As the data shows, it is clear that LEF-1-deficient T cells are able to produce a normal calcium flux in response to TCR engagement.

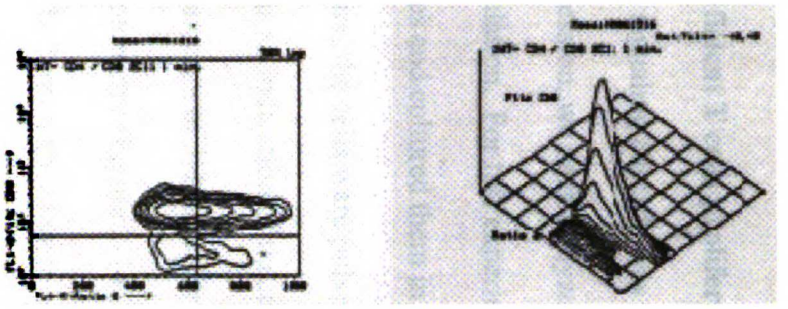
Figure 2-13 Calcium mobilization in LEF-1-deficient T cells. Two-color UV flow cytometry analysis of neonatal thymocytes using antibody against mouse CD8. Left panels represent resting cells and right panels contain activated T cells. Genotypes are listed for each row and data is displayed in duplicate, using both contour and relief mapping. The Y-axis of the probability plots is CD8 expression and the X-axis is ratio1/ratio2 measuring calcium-binding of Indo-1-AM.

Resting T cells

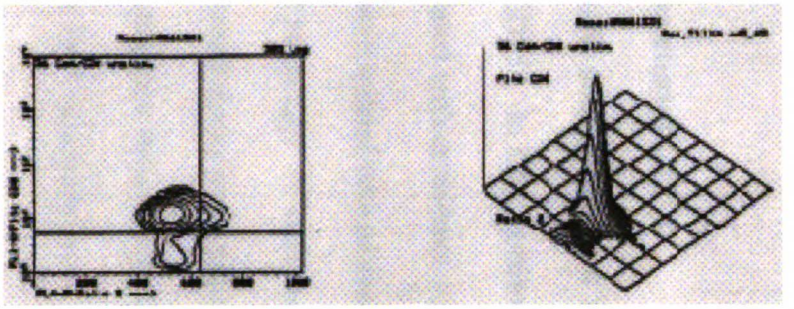
+2C11, I minute

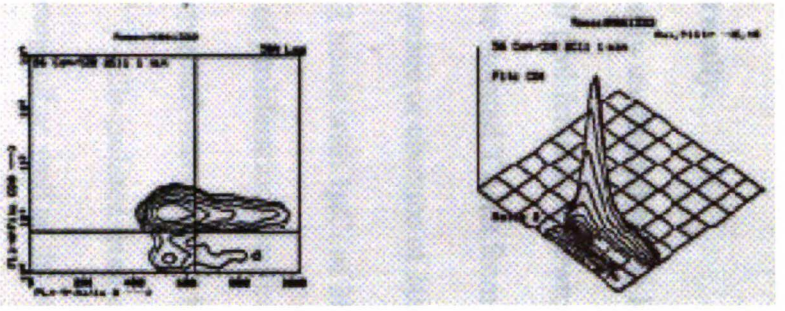
wild type

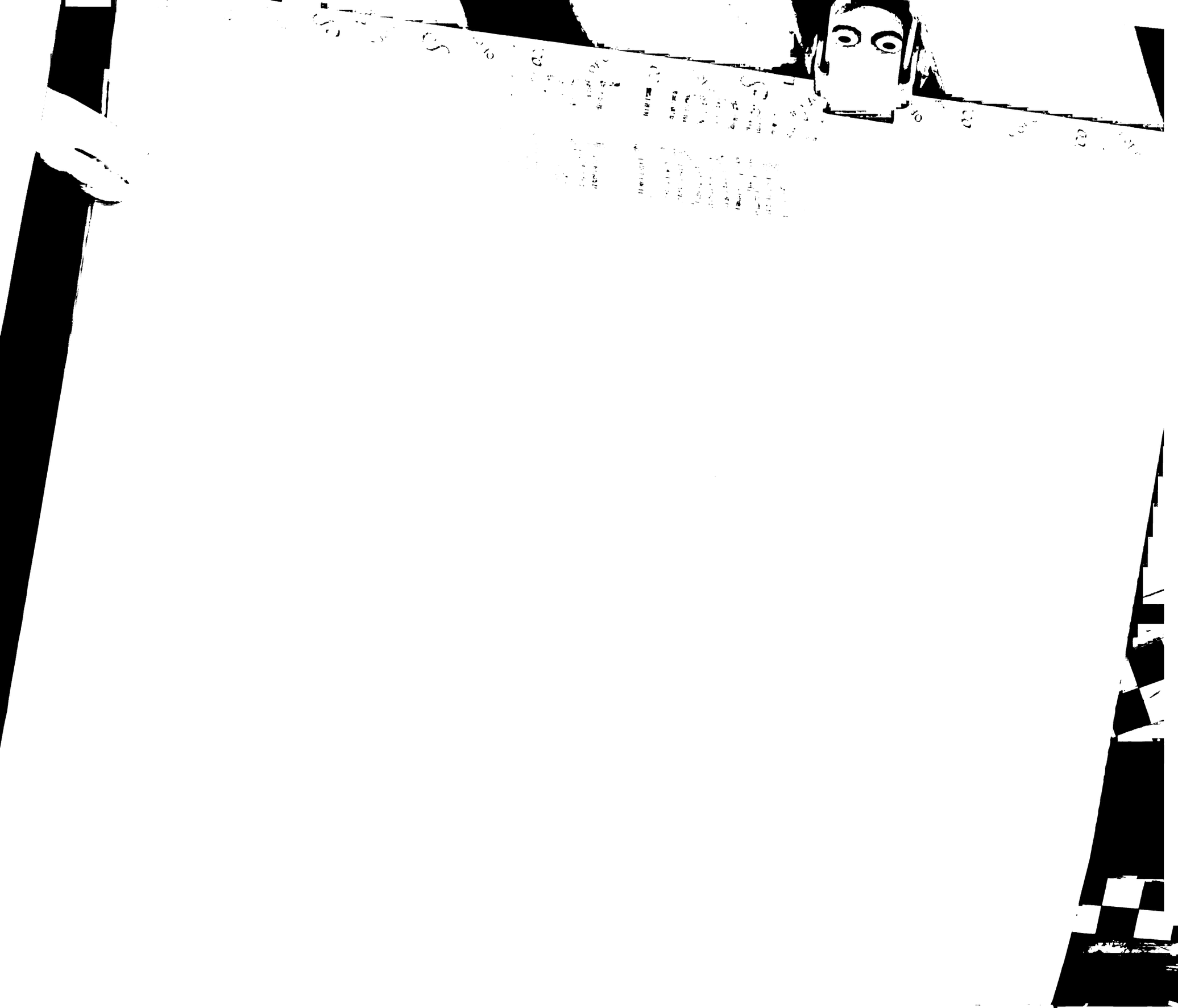




Lef1$^{-/-}$







Stimulated LEF-1-deficient T cells proliferate normally.

Since the early signaling cascade was apparently unaffected by the loss of functional LEF-1 expression, we next analyzed T cell proliferation, a downstream response to TCR stimulation. For this experiment, we isolated T cells from the thymus, spleen and lymph nodes and cultured them in the presence or absence of 10μg of 2C11. After 48 hours of culture, the cells were pulsed for 12 hours with 1μCi of 3H-thymidine. Uptake of the isotope is an indication of the amount of proliferation occuring in response to stimulation and the amount of 3H-thymidine incorporated was recorded by liquid scintillation spectrophotometry (Figure 2-14). *Lef1*$^{-/-}$ T cells isolated from either the thymus, the spleen or the lymph nodes were able to respond as well as the wild type cells in all cases, demonstrating that LEF-1 is not required for the T cell proliferative response.

Activated expression of CD154 does not require functional LEF-1.

Another aspect of TCR stimulation is the cellular differentiation of T cells which triggers the surface expression of the CD40 ligand, CD154. In this experiment, splenic T cells from adoptive transfer experiments were stimulated with or without 10μg of 2C11 for 12 hours in culture. After stimulation, the cells were stained with antibodies against mouse CD4 and CD154. Induced expression of CD154 on *Lef1*$^{-/-}$ T cells was recorded using flow cytometry (Figure 2-15) and the data collected shows no requirement for LEF-1 for the proper expression of CD154. These results together demonstrate that LEF-1 expression is not required for T cell differentiation or for T cell function.

Figure 2-14 Anti-CD3ε stimulation induces *Lef1*$^{-/-}$ T cells to proliferate. 3H-thymidine incorporation proliferation assay of T cells isolated from neonatal thymus, spleen and lymph node. Triplicate cultures of $1x10^6$ T cells from neonatal thymus, spleen and lymph node were stimulated with the 10μg/ml of the anti-CD3ε antibody 2C11. After 48 hours of culture at 37°C, cells were pulsed with 1μCi of 3H-thymidine and incubated 12 hours before harvesting. Radioactivity incorporation was measured by liquid scintillation spectrophotometry.

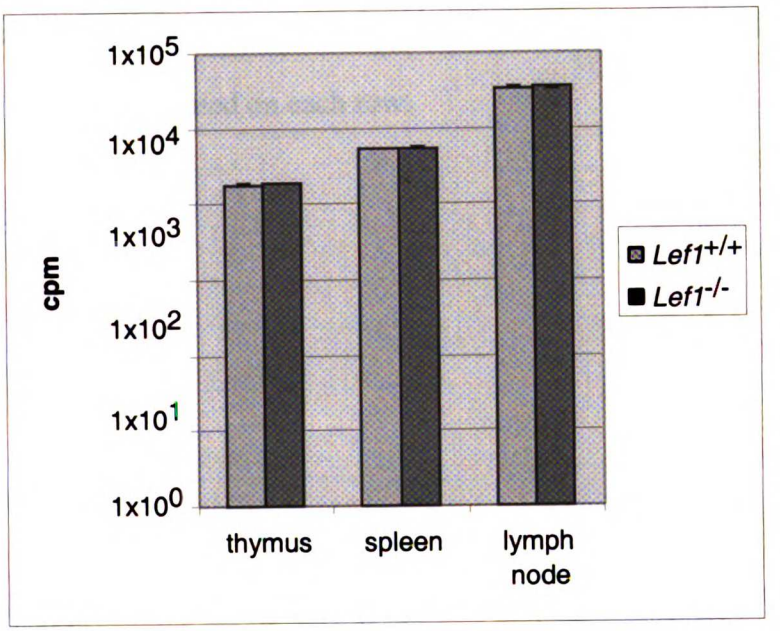
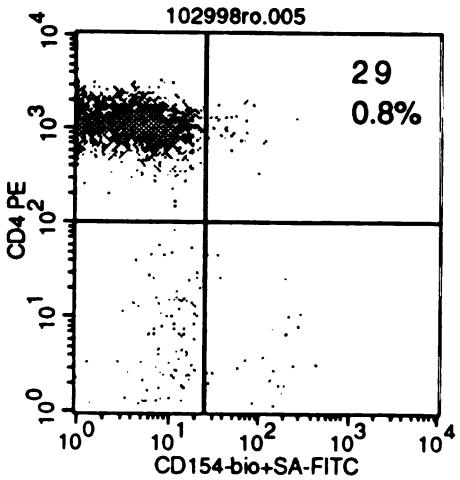

1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
cpm
thymus
spleen
lymph node
Lef1+/+
Lef1-/-

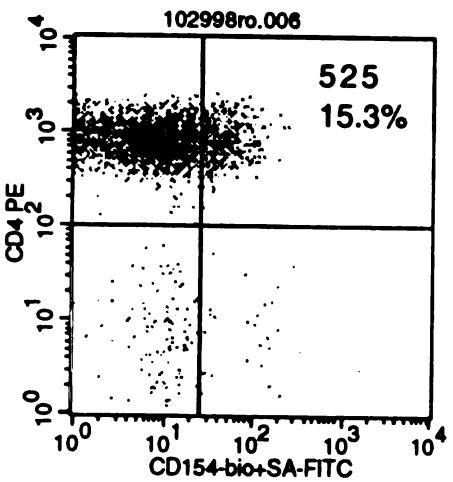
Figure 2-15 Activated expression of the antigen CD154 does not require LEF-1 expression. Two-color flow cytometry analysis of splenic T cells from adoptive transfer experiments using antibodies against mouse CD4 and CD154. The donor genotypes are indicated on each row. $2x10^6$ splenic T cells were isolated from adoptive transfer experiments and were cultured for 12 hours with or without the addition of 10μg 2C11. Cells were then stained with antibodies against mouse CD4 and CD154 and with propidium iodine. Shown is two-color flow cytometry analysis of splenic T cells from adoptive transfer experiments using antibodies against mouse CD4 and CD154. The donor genotypes are indicated on each row.

12 hr culture, no 2C11

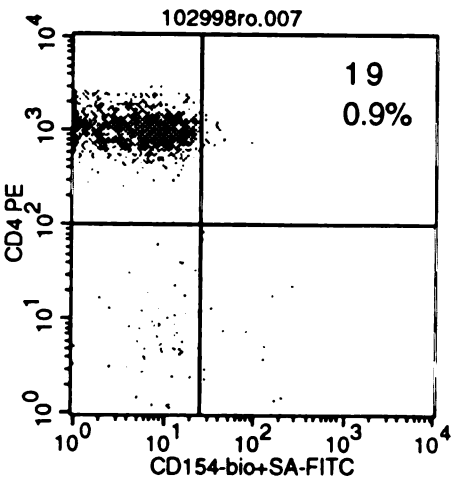
12 hr culture, +2C11

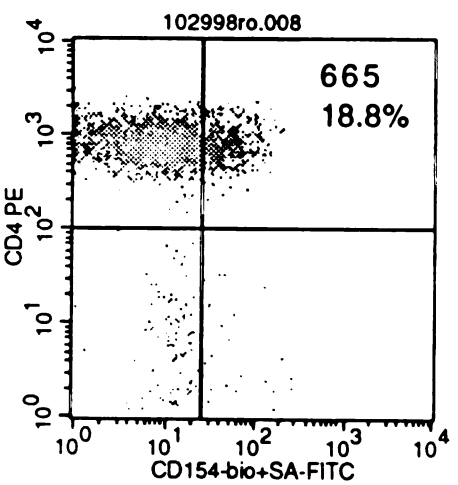
Lef1+/+

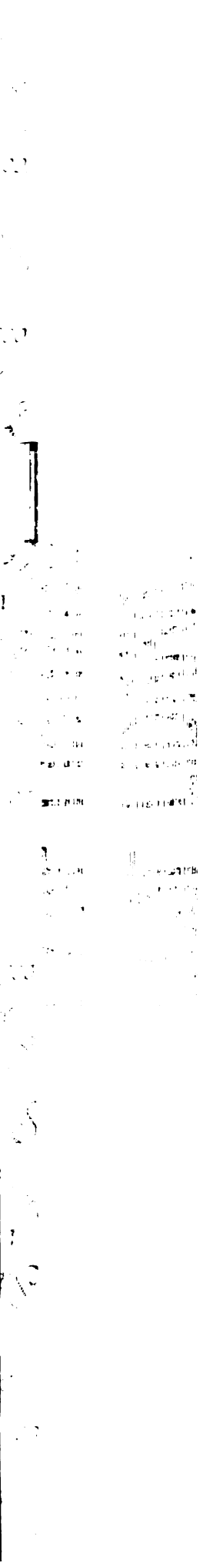




Lef1-/-







DISCUSSION

LEF-1-deficient mice were generated by targeted disruption of the HMG domain of the LEF-1 gene. While striking physiological defects are found during embryogenesis, the mutation of LEF-1 does not prevent the generation of a functional immune system. We show that LEF-1 deficient B lymphocytes are able to differentiate and rearrange the immunoglobulin heavy chain unimpeded, despite differences in the expression of the surface antigens BP-1 and MHC class II as well as the intracellular enzyme TdT. We also show that the B1a sub-population of B lymphocytes is more reliant on LEF-1 expression, however this appears to only be true in the mixed background of D3 and C57BL/6. Overall, we find that B cell function to antigenic challenge was unaffected in the LEF-1-deficient immune system. In T lymphocytes, despite the expression of LEF-1 in most early T cell sub-populations, T cell differentiation proceeds through all known steps with no block in development. Mature *Lef1*$^{-/-}$ T cells are able to generate a calcium flux, proliferate and differentiate normally in response to T cell stimulation. Additionally, the activity of the only fully characterized target of LEF-1 activity, the TCRα gene, appears to not require the expression of functional LEF-1 to allow transcription. These data suggest that the expression of LEF-1 is not required during B and T lymphogenesis.

LEF-1 is expressed in a restricted fashion in the pro-B cell fractions of differentiating B lymphocytes. We find that there are abnormalities caused by the loss of functional LEF-1. The reduced expression of the surface antigen BP-1 and the elevated levels of expression of MHC class II and TdT would indicate that these are potential targets of LEF-1. There is no evidence that any of these genes are directly regulated by

LEF-1, nor is there any previous data that would predict this result in the *Lef1*$^{-/-}$ mice. As striking as these differences are, they are not enough to disrupt B cell differentiation as pre-B cell and mature B cell populations are present in the LEF-1-deficient mouse. The analysis of immunoglobulin heavy chain V(D)J rearrangement further demonstrates that LEF-1 expression does not influence the B cell repertoire and the antigenic challenge in the adoptive transfer SCID mice shows that LEF-1-deficient B cells function normally. A potential explanation for the normal B lymphocyte differentiation seen in LEF-1-deficient mice would be a redundancy with another HMG family member, similar to what is seen in the T cell lineage [24]. The HMG family member TCF-4 is an intriguing candidate because it is expressed in the same immature B cell populations as LEF-1 (TR ref). From these data, we can conclude that LEF-1 does play a role during B cell differentiation, but that the expression of LEF-1 is not essential for the process to reach completion.

Unlike the conventional B cells, a clear defect is seen in the production of the CD5^{+} B1 cell population. This defect, however, appears to be strain specific. In this first set of adoptive transfer experiments using mixed background donor tissue, 18 SCID mice received wild-type fetal liver and 36 received LEF-1-deficient fetal liver. These experiments were performed in eight cohorts of four to twelve animals each over the span of a year. The results from each experiment were consistent in that the animals that received wild-type donor tissue were able to produce the CD5^{+} B1 population and the animals that received *Lef1*$^{-/-}$ fetal liver could not. After backcrossing the animals fifteen generations to the C57BL/6 strain, the experiment was repeated and the results directly contradicted the previous data in that both genotypes were now able to produce the CD5^{+}

B1 cells. This second set of experiments was performed with two cohorts of ten animals each. To address the question of whether this difference was due to a strain difference, we initiated a backcross to the mouse lineage from which the D3 embryonic cell line was derived, the 129/SvJ strain. At the time that these studies were completed, we had successfully backcrossed the *Lef1*$^{-/-}$ line by three generations. The most striking result was an immediate increased viability of the LEF-1-deficient mice. In the F1 mixed background, the LEF-1 deficient mice would live up to 21 days with an average life span of 10 days. During the period in which we backcrossed the mice to the C57BL/6 strain, we noted that the mice usually died within the first 24 hours after birth. Within the first backcross to the 129/SvJ strain, LEF-1 deficient mice would live to 3 days and the second generation of mice could survive as long as two weeks. These observations would certainly suggest that the relative need for functional LEF-1 is different between the two strains of mice.

Several possible explanations exist for how such a strain difference could arise. These include the presence or absence of other HMG family members (TCF), transcriptional co-factors (ALY), or post-translational modifiers. Recently, the CD5^{+} B1 cell population itself has been shown to express different levels of the surface antigen B220 depending on the strain observed [25], providing evidence that the population behaves differently in between mouse strains. An example of a more well studied immunological strain difference in mice is the mouse model for *Leishmania Major* infection (reviewed in [26]). Most strains of mice present a healer phenotype and are able to ward off an infection with *L. Major*, but the BALB/C strain is unable to defend

itself [27]. The genetic cause of this susceptibility has been linked to chromosome 11 [28, 29] but the specific gene remains as yet unidentified.

Perhaps the most unexpected result from this study was the normal T lymphocyte differentiation in LEF-1-deficient animals. Previous *in vitro* studies have shown conclusively that LEF-1 is required for the function of the TCRα minimal enhancer [5, 20]. In addition, LEF-1 has been shown to be expressed during multiple stages of early T cell differentiation, reaching the highest level of expression at the same stage as when the TCRα gene begins to be transcribed [19]. As TCRα expression is required for T cell differentiation [22], it was possible that the activity of LEF-1 was required for the generation of T cell lineage. For these reasons, it is somewhat surprising that the LEF-1-deficient mice do not display any detectable abnormality in T cell differentiation, T cell function or TCRα expression. An explanation that would not invalidate any of the *in vitro* studies would be that LEF-1 acts in a functionally redundant manner with another protein. One such candidate for this scenario would be the related transcription factor TCF-1 that is expressed in an overlapping pattern with LEF-1 in differentiating thymocytes [19] and contains an identical HMG DNA-binding domain. While LEF-1 has been shown to activate transcription on the TCRα minimal enhancer to a higher degree than TCF-1 [30], TCF-1 is expressed at higher levels than LEF-1 in T cells [19]. The redundant function of these two transcription factors is explored in the next chapter, which details studies on mice deficient for both HMG family members.

This study details the immunological phenotypes of the LEF-1 deficient mouse. LEF-1 is required in conventional B lymphocytes for the correct expression of BP-1, MHC class II and TdT, but not for B cell differentiation or function. In addition, in a

strain-specific manner, LEF-1 is required for the $CD5^+$ B1 cell population. Finally, the absence of LEF-1 was not enough to cause any detectable change in T lymphocyte differentiation or function, nor did it cause a reduction in the expression of the target gene TCRα. What remains unknown is how LEF-1 influences the transcriptional regulation of the newly identified targets described here. The determination of the answer to this question is the next logical extension of this work.

Materials and methods

Mice

C57BL/6, BALB/c SCID, and 129/SvJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained in the Animal Care Facility at the University of California, San Francisco (UCSF). LEF-1 deficient mice were generated and screened as previously described [1]. All experimental adult animals were between the ages of 8-12 weeks of age.

Flow cytometry and adoptive transfers

Flow cytometry was performed using a FACStar plus (Becton Dickinson), a FACSCalibur (Becton Dickinson) or a modified, dual laser FACS II (FLASHER, Stanford Shared FACS Facility, Stanford) as described [24]. Adoptive transfers were performed using BALB/c SCID mice (Jackson Laboratories) as recipients in all experiments following the protocol as described [24].

Antibodies

Directly conjugated antibodies specific for mouse B220, CD3ε, CD4, CD8, TCRβ, and TCRγ/δ were obtained from Caltag (San Francisco, California). Directly conjugated and unconjugated antibodies specific for mouse CD3ε, CD5, CD43, IgD_b, IgM_b, I-A and MacI were obtained from Pharmingen (La Jolla, California).

Proliferation assays

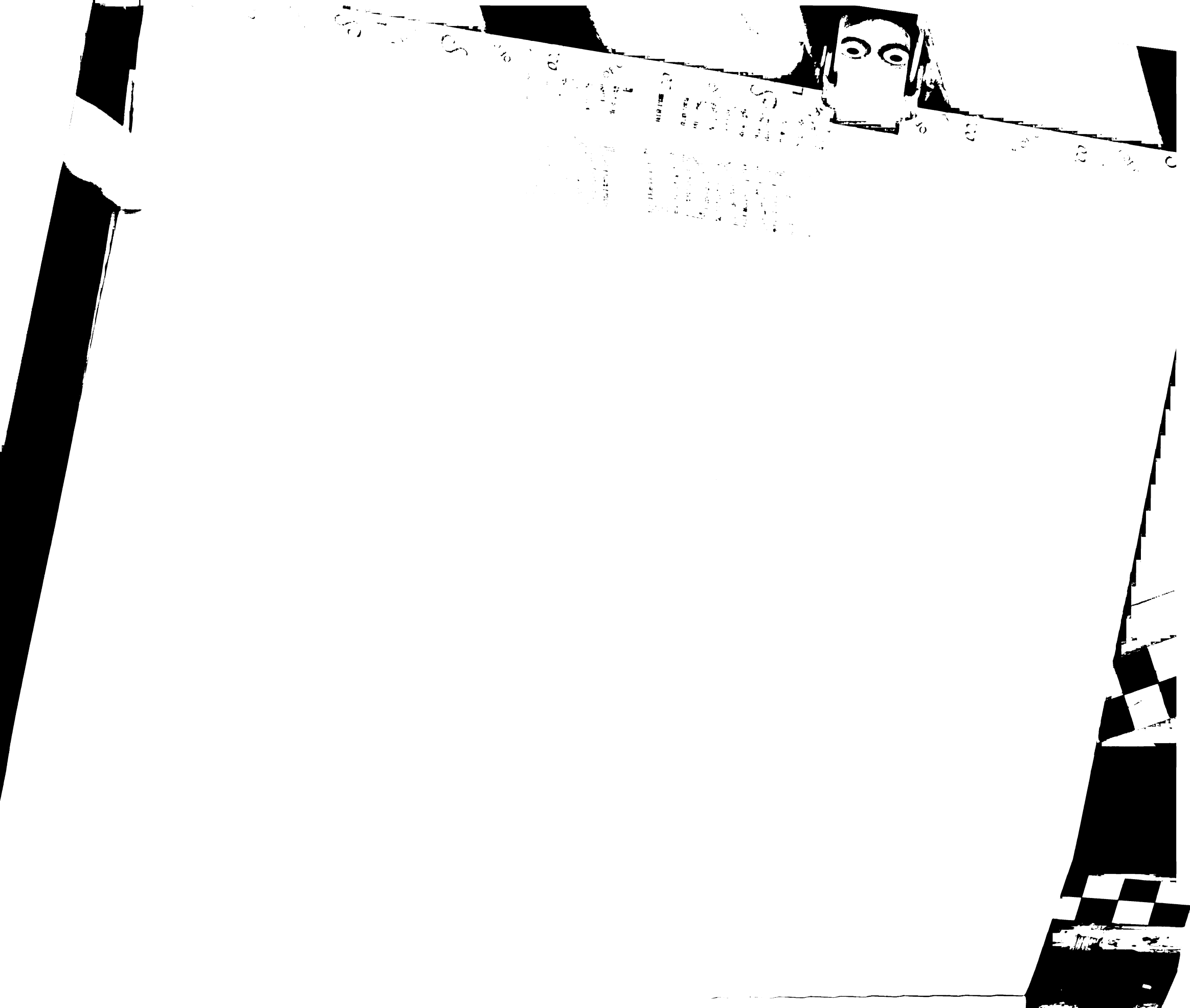
Triplicate cultures of $1x10^6$ cells were incubated with 200µl of medium (RPMI with 10% fetal calf serum, penicillin/streptomycin, and 2-mercaptoethanol) in 96-well flat-bottomed microtiter plates. T cells were stimulated with the 10µg/ml of the anti-CD3e antibody 2C11 (Pharmingen) or with PMA and ionomyicin. B cells were stimulated with 10µg/ml of an anti-IgM antibody (Pharmingen). After 48 hours of culture at 37°C, cells were pulsed with 1µCi of 3H-thymidine (NEN) and incubated 12 hours before harvesting. Radioactivity incorporation was measured by liquid scintillation spectrophotometry.

Calcium release assay.

$2x10^7$ thymocytes were loaded with 3µM Indo-I-AM in 1ml PBS for 20 minutes at 37°C. The concentration of cells was lowered to $2x10^6$/ml with the addition of PBS and the incubation continued for another 20 minutes at 37°C. The cells were then stained with antibodies against mouse CD4 and CD8 for 20 minutes on ice. After staining, the cells were incubated at 37°C for 10 minutes. Cells were analyzed by flow cytometry first without stimulation and then every 30 seconds after the addition of 10µg of 2C11. As a control, cells were stimulated non-specifically with the calcium ionophore ionomycin.

CD154 expression assay.

$2x10^6$ splenic T cells were isolated from adoptive transfer experiments. The T cells were cultured for 12 hours in 2ml of medium (RPMI with 10% fetal calf serum, penicillin/streptomycin, and 2-mercaptoethanol) with or without the addition of 10mg



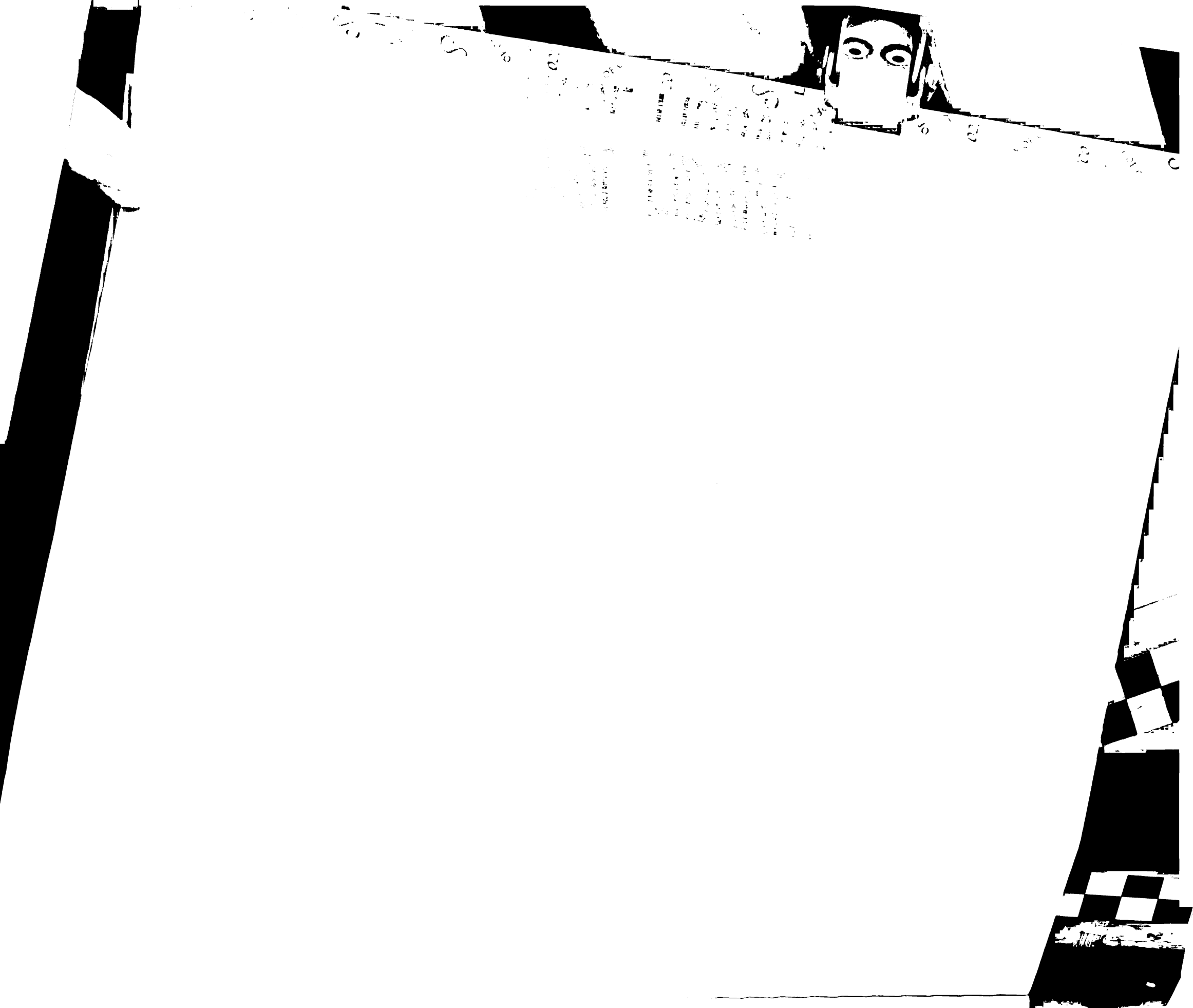
2C11. Cells were then stained with antibodies against mouse CD4 and CD154 and with propidium iodine. $CD4^+/PI^-$ cells were analyzed by flow cytometry for the expression of CD4 and CD154.

Single cell V(D)J rearrangement analysis.

This assay was originally described in [6] and modified in [7]. Briefly, single B220+ cells were sorted using a FACStar plus (Becton Dickinson) into a 96-well plate containing 10μl of a lysis buffer with Proteinase K. Cells were lysed for one hour at 55°C, followed by an inactivation step of 95°C for 5 minutes. Subsequently, two rounds of nested PCR were performed using 26 different primers [7, 31, 32]. PCR products were analyzed by electrophoresis on a 2% agarose gel and Vh usage was scored by the presence or absence of specific products. Some V(D)J PCR products were cloned and sequenced to determine the presence of N-region addition by the enzyme TdT.

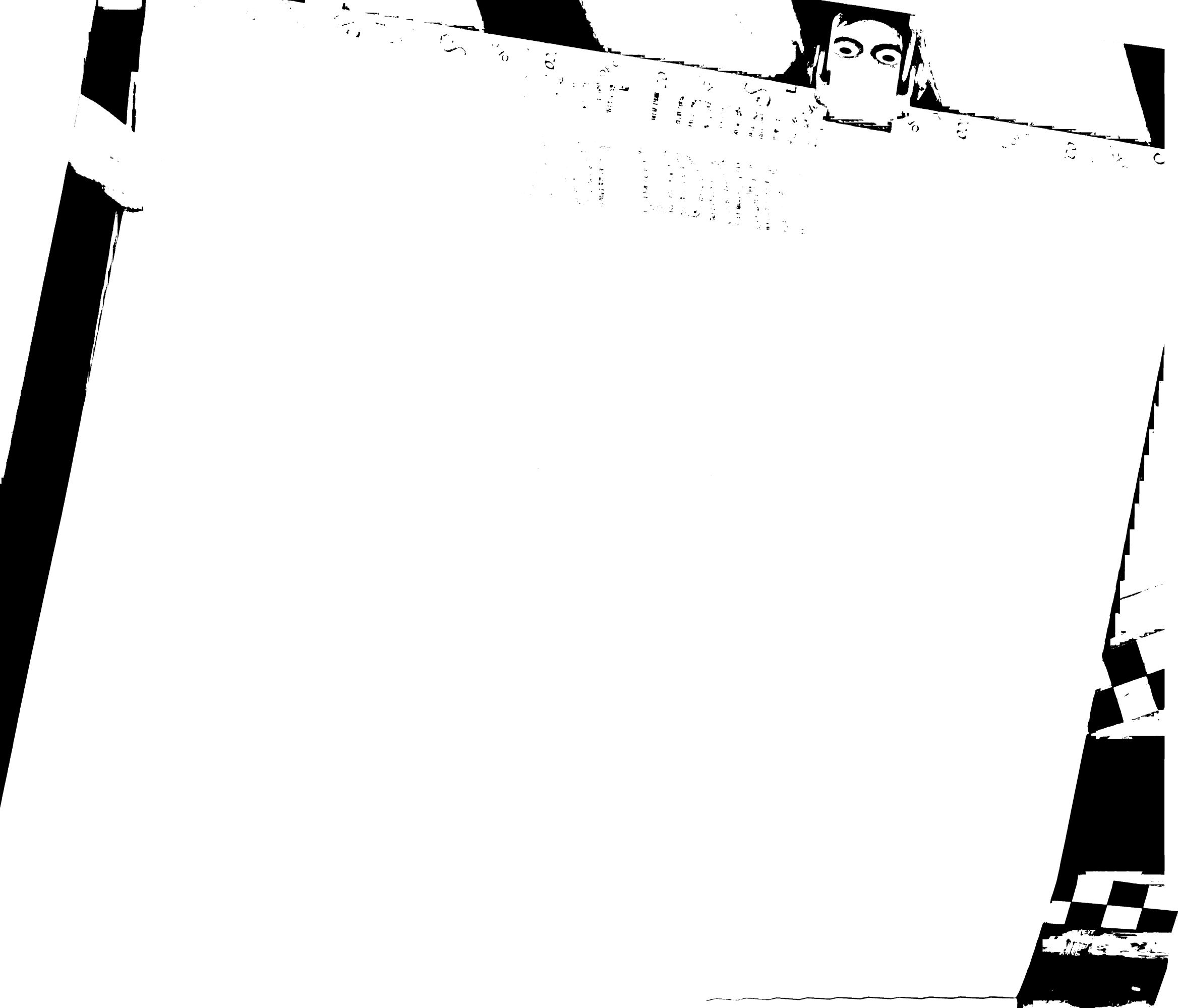
Antigenic challenge of adoptive transfer experiments.

SCID adoptive transfers were screened for the presence of B and T cells in peripheral blood by flow cytometry. Only successful adoptive transfers were used in this protocol. Before immunization and before each booster reimmunization, 100 μl of serum was collected from each animal. 50 μg of antigen (either TNP-KLH or NIP-Ficoll) with 2mg of alum was injected I.P. on days 0, 14, 21, 28 and 35. Samples were stored at –80°C after collection and analysis was performed on all time points simultaneously.



Serum analysis.

96 well flat-bottom plates (Falcon 3077) were coated with 0.5μg of antigen (either TNP-BSA or NIP-BSA) in 50μl of PBS overnight at 4°C. The wells were then blocked with 150ml of PBST (PBS + 0.05% Tween) with 10% FCS for 1 hour at room temperature. The plates were next washed with PBST three times. Samples composed of 3μl of serum diluted with 47μl of PBS were added to each well. All serum analysis was performed in triplicate. Samples were bound for 10 hours at room temperature. After binding, plates were again washed 3 times. Secondary antibody (anti-IgG1-biotin) in 50μl of PBST with 1% BSA was next added and bound for 1 hour at room temperature. After another wash step, SA-alkaline phosphatase in 75μl of PBST with 1% BSA was added and bound for 1 hour at room temperature. A final wash step was performed and substrate was added. Plates were read on a Spectramax plate reader using Softmax analysis software.



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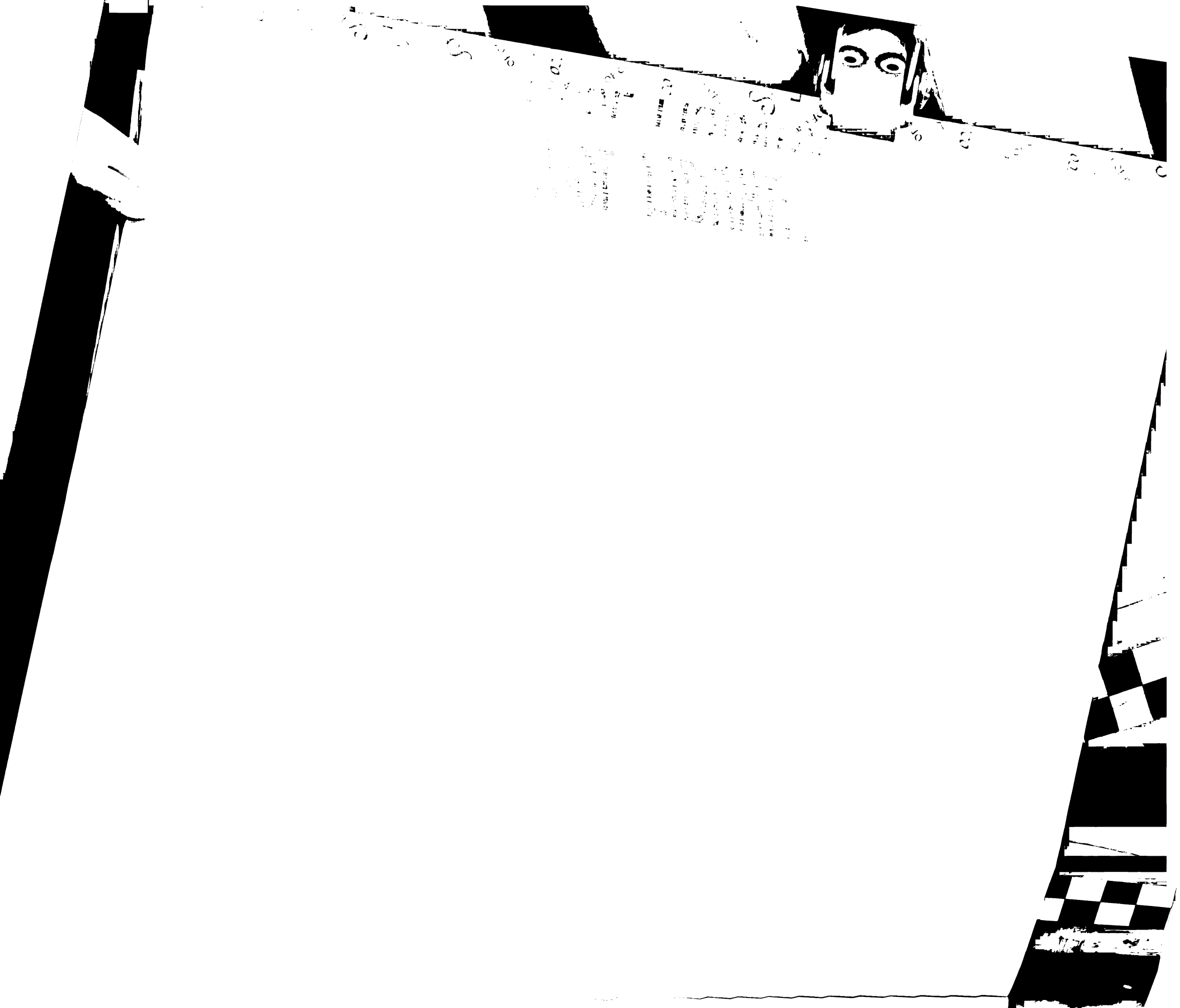

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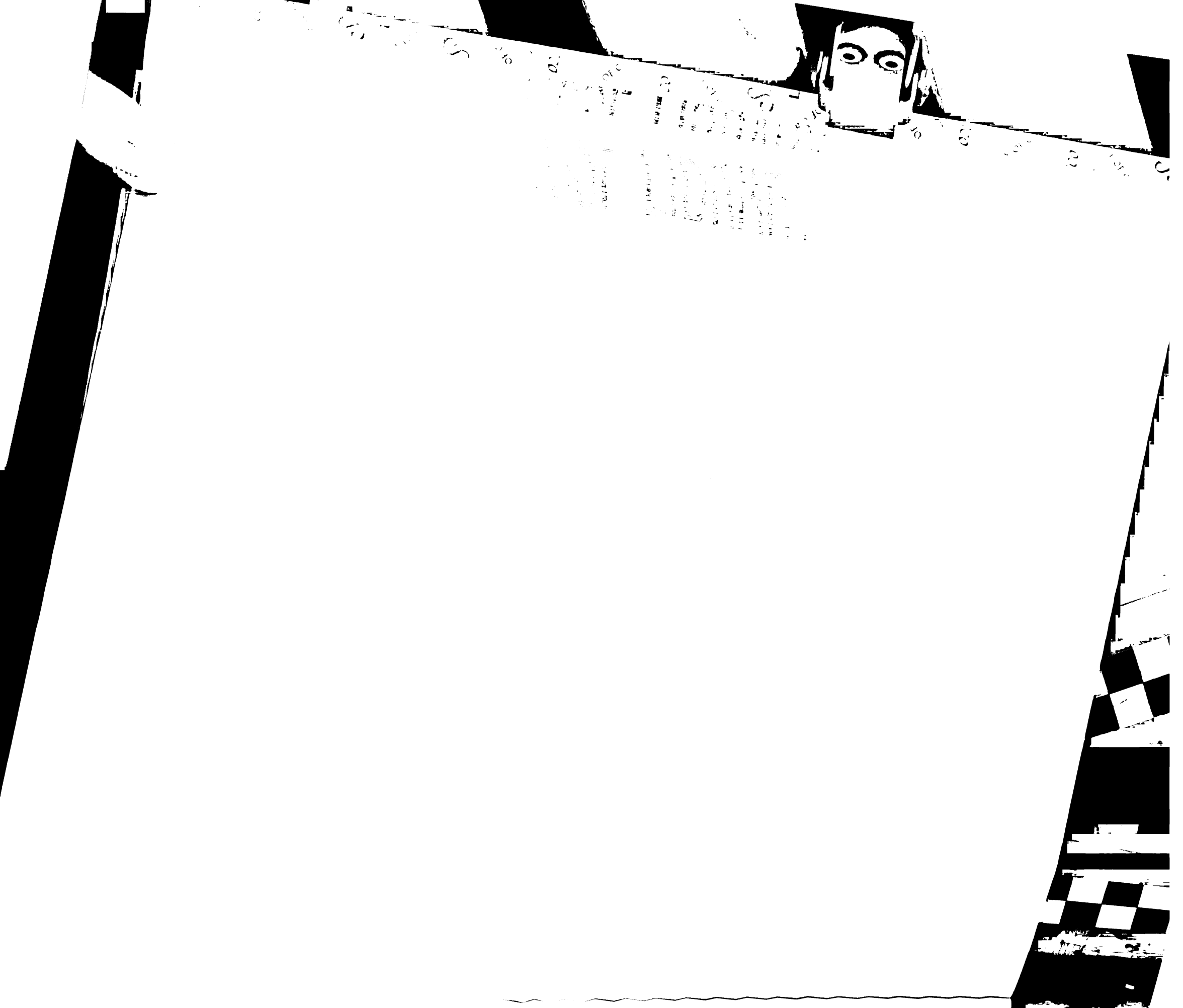
Chapter 3

Redundant regulation of T cell differentiation and TCRα gene expression by the transcription factors LEF-1 and TCF-1



SUMMARY

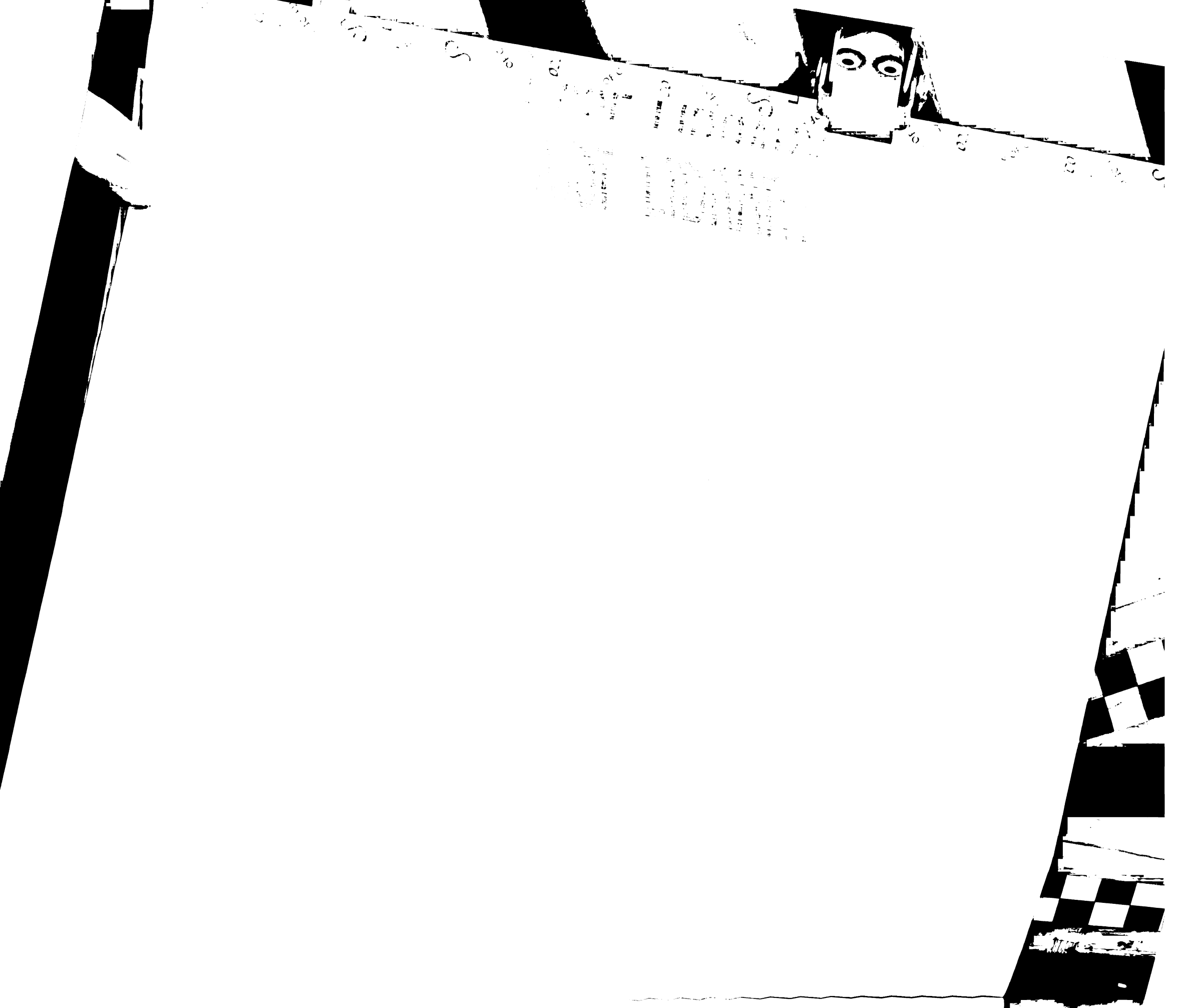
Lymphoid enhancer factor 1 (LEF-1) and T cell factor 1 (TCF-1) are closely related transcription factors that are both expressed during murine T cell differentiation and regulate the T cell receptor α (TCRα) enhancer in transfection assays. Targeted gene disruption of either the *Tcf1* or *Lef1* gene in mice did not affect TCRα gene expression and resulted in an incomplete or no defect in thymocyte differentiation. Here, we examine a potential redundancy of these transcription factors by analyzing double mutant mice. In fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice, α/β T cell differentiation is completely arrested at the immature CD8^{+} single positive (CD8^{+} ISP) stage and is markedly impaired at an earlier stage. In addition, we find that sorted CD8^{+} ISP cells from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice express TCRβ but show a severely reduced level of TCRα gene transcription. Taken together, these data show that LEF-1 and TCF-1 are redundant in the regulation of T cell differentiation and gene expression.

RESULTS

Redundant regulation of α/β T cell differentiation by LEF-1 and TCF-1 at the immature CD8$^+$ single positive stage.

To examine whether LEF-1 and TCF-1 regulate T cell differentiation and gene expression in a redundant manner, we generated mice carrying targeted mutations in both genes. Given the perinatal lethality of the *Lef1*$^{-/-}$ mice [1], we crossed homozygous *Tcf1(V)*$^{-/-}$ mutant mice for a cross with heterozygous LEF-1 deficient mice to avoid the possibility of an early embryonic lethality. Double heterozygous *Lef1*$^{+/-}$*Tcf1(V)*$^{+/-}$ mutant mice were further crossed to generate homozygous double mutant mice who were born at normal frequency but died at birth.

T cell differentiation in the thymi of mutant mice deficient in both LEF-1 and TCF-1 was analyzed in fetal thymic organ cultures (FTOC) (Ramsdell, 1992). We removed the thymus from E17.5 embryos which normally contain double negative and double positive immature T cells, but lack the mature CD4$^+$ and CD8$^+$ single positive cells (Ramsdell, 1992). We cultured the organs for seven days to allow further differentiation and generation of the mature T cells. The genotypes of the embryos were determined by DNA blot analysis using tail genomic DNA (data not shown). The viability of cells within the organ cultures was monitored by FACS analysis using propidium iodide. Non-viable cultures were excluded and all experiments were performed in triplicate. Differentiation of thymocytes in FTOC was examined by analysis of surface expression of CD4 and CD8, TCRβ and TCRγ/δ, and CD25 and CD44. The total number of thymocytes in thymic organ cultures from *Tcf1(V)*$^{-/-}$ embryos was approximately 50% of the number observed in cultures from normal mice. This



number of thymocytes was reduced further by a factor of 14 in the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ organ cultures (Table 3-1). Analysis of thymocytes for surface expression of CD4 and CD8 indicated that the cultures from *Lef1*$^{-/-}$*Tcf1*$^{+/+}$ mice contained differentiating T cell populations similar to those of the control cultures (Figure 3-1). Consistent with the phenotype in one month-old *Tcf1*$^{-/-}$ mice [2], fetal thymic organ cultures from both *Tcf1(V)*$^{-/-}$ and *Tcf1(VII)*$^{-/-}$ mutant mice show an increase in the relative number of CD8$^+$ cells and the *Tcf1(VII)*$^{-/-}$ cultures show a relative decrease in the number of CD4$^+$ cells. This defect in T cell differentiation is significantly exacerbated in the *Lef1*$^{+/-}$*Tcf1(V)*$^{-/-}$ organ cultures. In fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice, T cell differentiation is almost completely blocked and contains virtually no CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ T cells. Residual CD4$^+$CD8$^-$ cells (1.4%) present in the fetal thymic organ cultures from the homozygous double mutant mice express the NK1.1 surface antigen which is characteristic of NK cells (data not shown) and thus appear not to be conventional mature CD4$^+$CD8$^-$ α/β T cells. The abundant population of CD4$^-$CD8$^+$ T cells most likely represents immature CD8$^+$ single positive cells because the majority of this population does not display detectable surface expression of TCRβ (see Figure 3-2).

To examine whether the deficiency of LEF-1 and TCF-1 affects both α/β and γ/δ T cell lineages, we analyzed the thymocytes for the expression of TCRβ and TCRγ/δ. Abundant TCRβ surface expression was detected in fetal thymic organ cultures from mice carrying either single gene disruption whereas the number of TCRβ-expressing cells in the homozygous double mutant cultures was reduced to only 6% of the total thymus (Figure 3-2). The percentage of cells expressing TCRγ/δ on their cell surface is

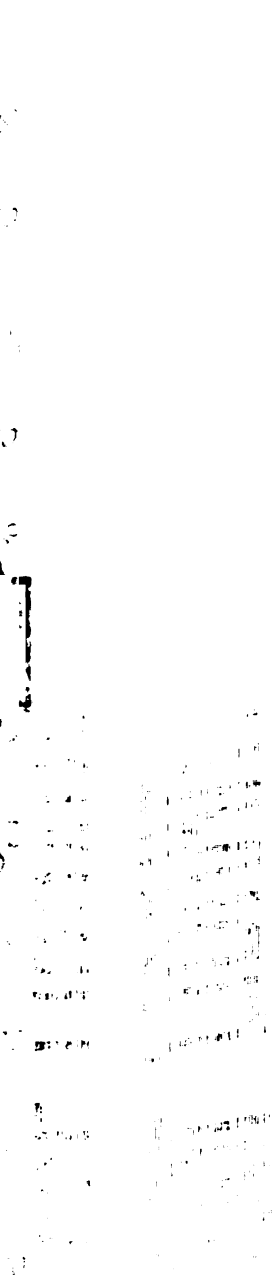


Table 3-1. Summary of fetal thymic organ culture experiments

genotype	# of cultures	T cells	total thymocytes	% T cells
Wild Type	7	$2.76x10^5$	$3.7x10^5$	74%
Lef1$^{-/-}$*Tcf1*$^{+/+}$	6	$1.89x10^5$	$2.7x10^5$	69%
Lef1$^{+/+}$*Tcf1(V)*$^{-/-}$	4	$1.26x10^5$	$1.7x10^5$	74%
Lef1$^{+/-}$*Tcf1(V)*$^{-/-}$	5	$1.01x10^5$	$1.2x10^5$	84%
Lef1$^{-/-}$*Tcf1(V)*$^{-/-}$	5	$8.48x10^3$	$1.2x10^4$	68%

Thymus was isolated from E17.5 embryos and cultured at 37ϒC for seven days in supplemented RPMI. The numbers of total thymocytes were determined by flow cyotmetry, using size and complexity gating and propidium iodine uptake to identify live cells. T cells were identified by antibody staining against Thy1.2, CD4 and CD8. The number of T cells in *Tcf1*$^{-/-}$ fetal thymic organ cultures is reduced by a factor of 2 to 3 relative to control cultures and the number of T cells in the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures is reduced by a factor of 30. Standard deviation of the number of total thymocytes was less than 50% and the standard deviation of % T cells per culture was below 5%.

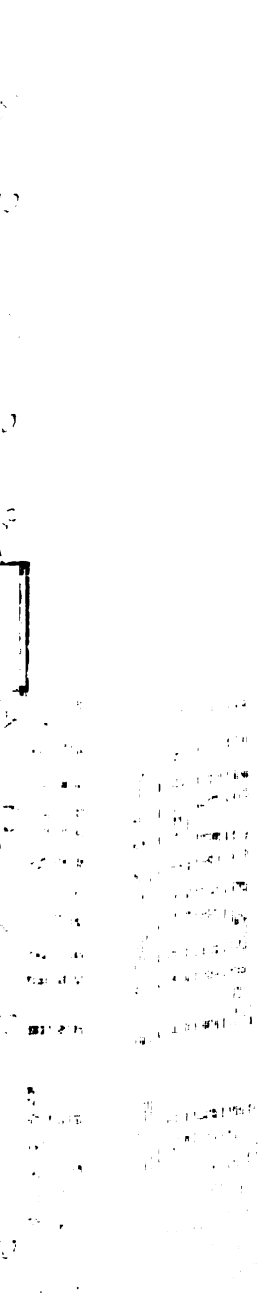
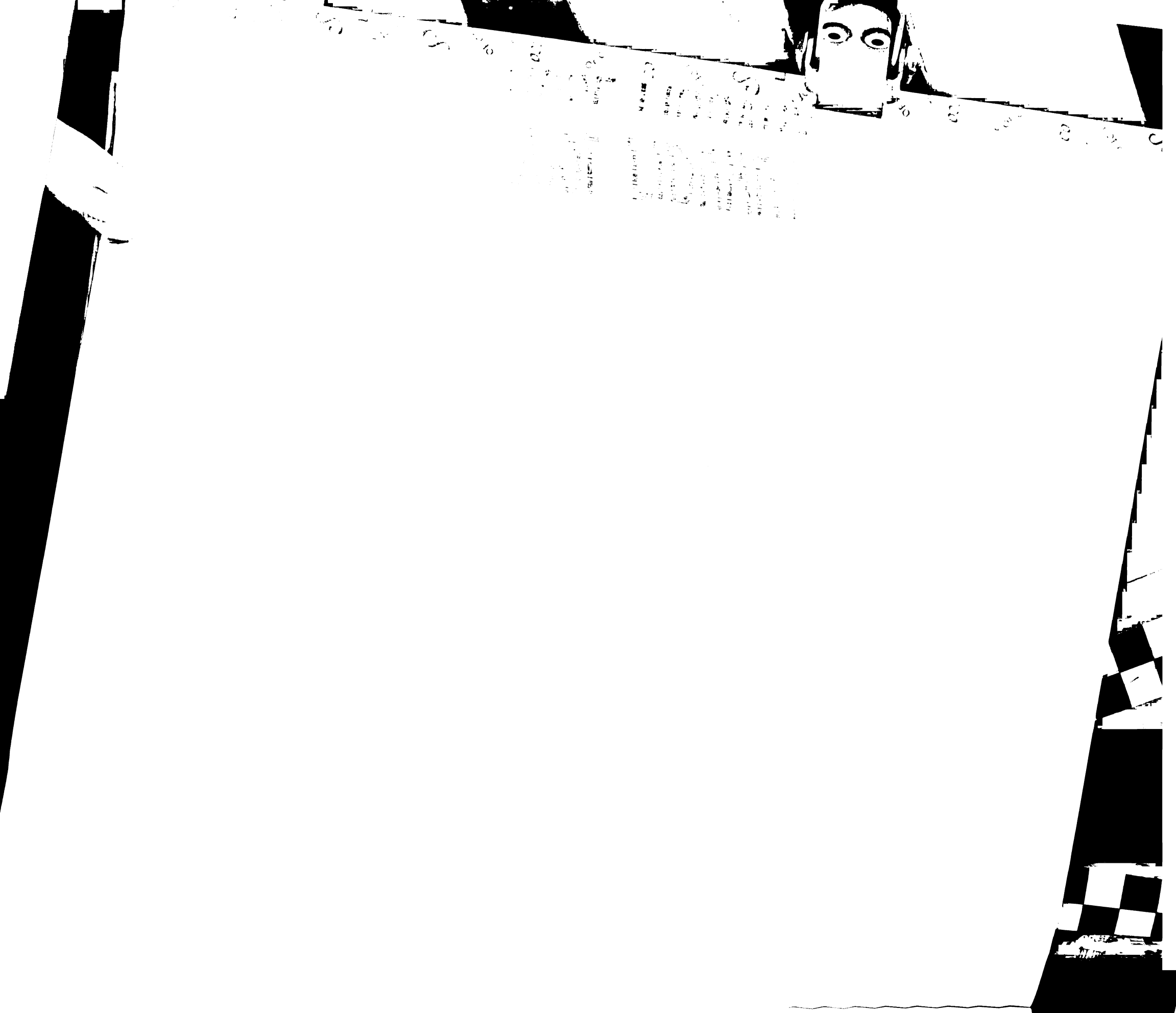


Figure 3-1. Expression of functional LEF-1 and TCF-1 is important for the development of double positive T cells. Two-color flow cytometry analysis of E17.5 fetal thymic organ cultures, after seven days of culture, using antibodies against mouse CD4 and CD8. Percentages of cell populations are calculated from total viable lymphocytes by gating according to size (forward scatter) and complexity (side scatter). The genotypes of the organ cultures are indicated above each panel. Each panel is representative of a minimum of three independent organ cultures.



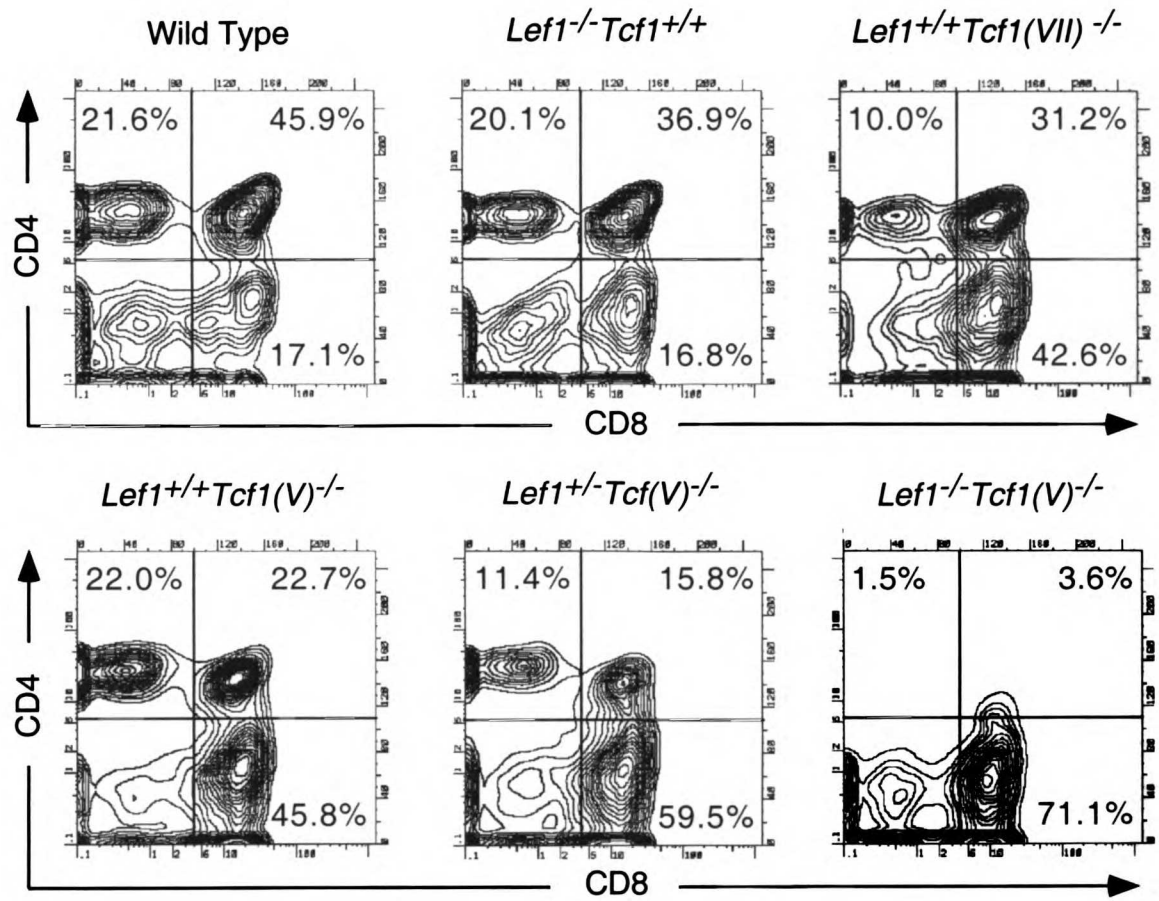
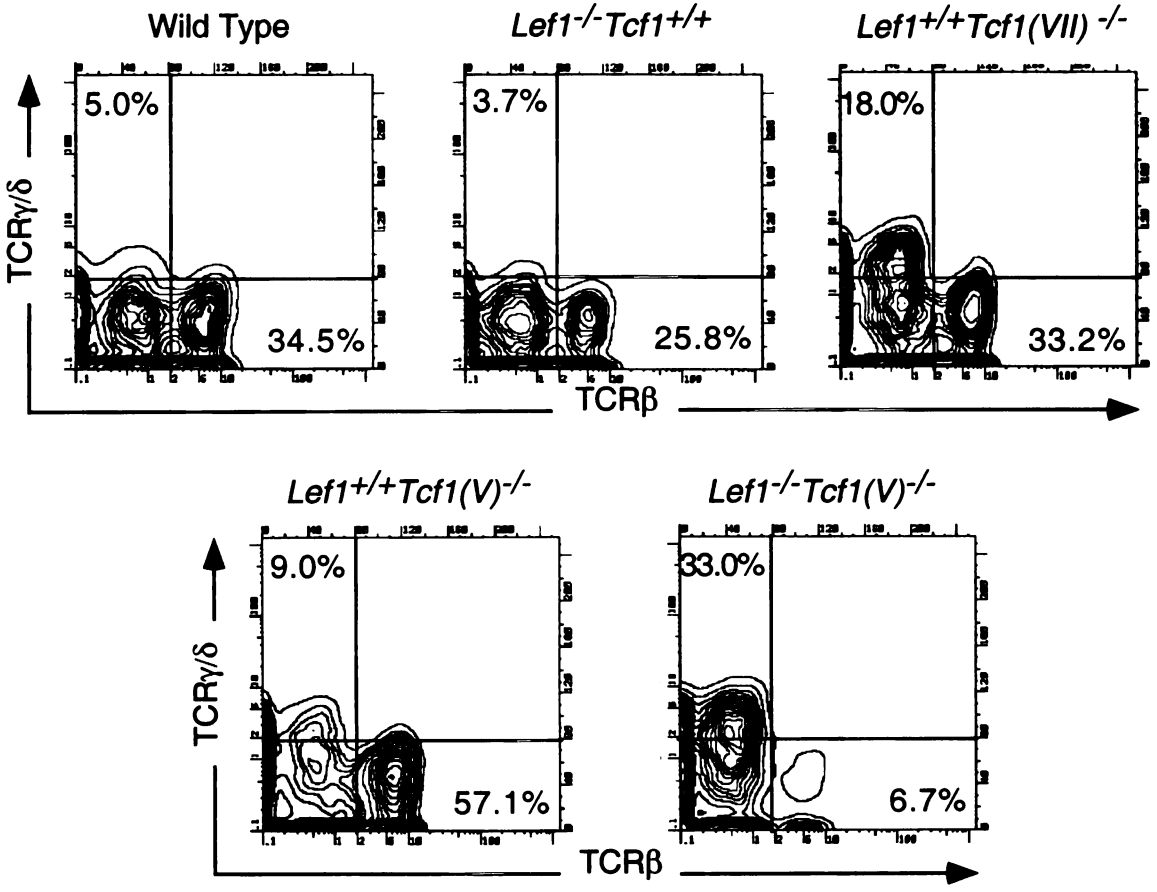
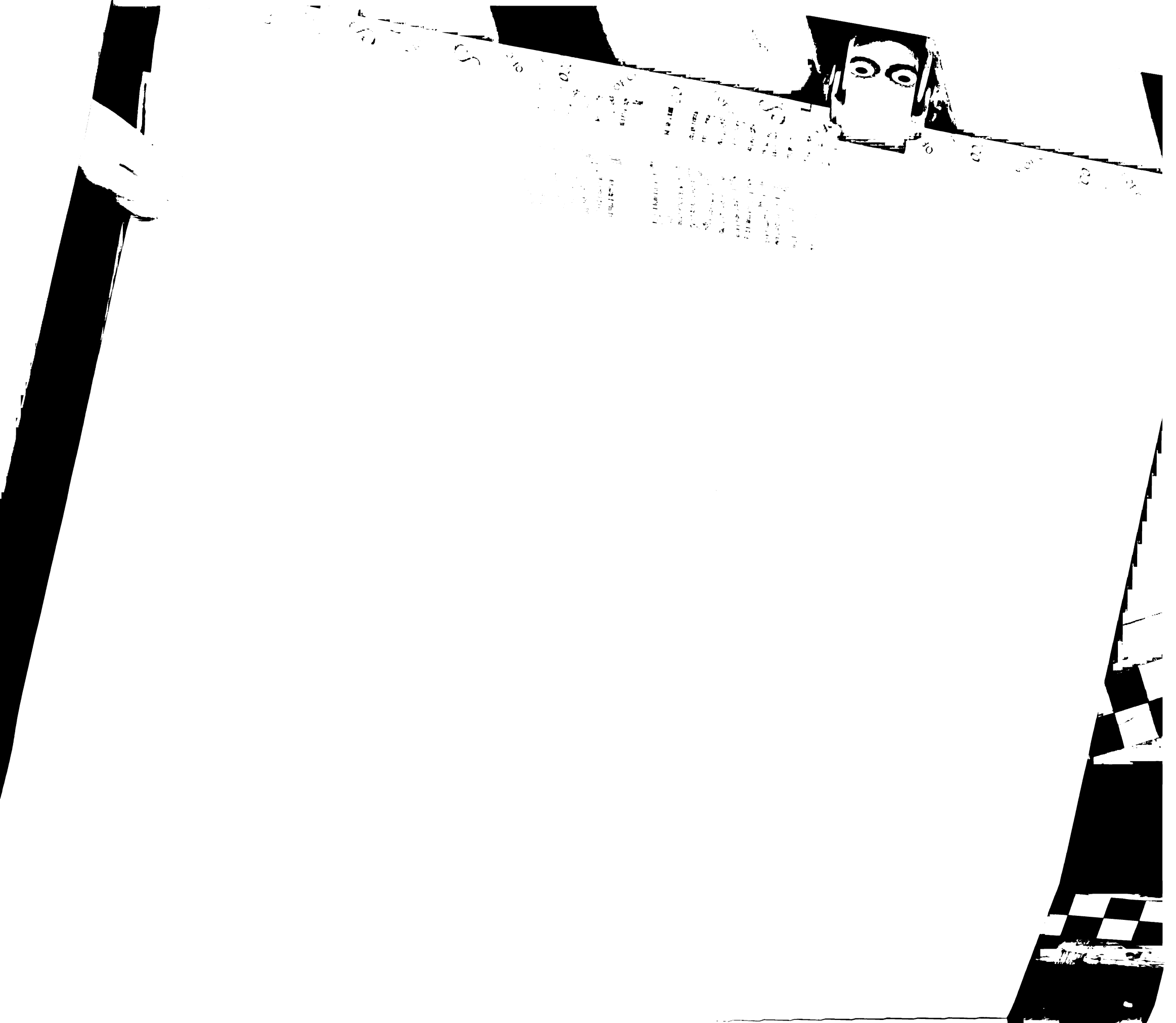

Wild Type
Lef1-/-Tcf1+/+
Lef1+/+Tcf1(VII) -/-
21.6%
45.9%
17.1%
20.1%
36.9%
16.8%
10.0%
31.2%
42.6%
CD4
CD8
Lef1+/+Tcf1(V)-/-
Lef1+/-Tcf(V)-/-
Lef1-/-Tcf1(V)-/-
22.0%
22.7%
45.8%
11.4%
15.8%
59.5%
1.5%
3.6%
71.1%
CD4
CD8

Figure 3-2. T cell differentiation in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures is blocked in α/β but not γ/δ T cells. Two-color flow cytometry analysis of E17.5 fetal thymic organ cultures, after seven days of culture, using antibodies against mouse TCRβ and TCRγ/δ. γ/δ T cells are determined as cells that are positive only for TCRγ/δ staining and α/β T cells are determined as cells that are positive only for TCRβ staining.


Wild Type
Lef1-/-Tcf1+/+
Lef1+/+Tcf1(VII) -/-
5.0%
34.5%
3.7%
25.8%
18.0%
33.2%
TCRγ/δ
TCRβ
Lef1+/+Tcf1(V)-/-
Lef1-/-Tcf1(V)-/-
9.0%
57.1%
33.0%
6.7%
TCRγ/δ
TCRβ



increased in cultures from *Tcf1(V)*$^{-/-}$ and *Tcf1(VII)*$^{-/-}$ mutant mice by a factor of two- and five-fold respectively. In *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures this effect is further accentuated, increasing the percentage of γ/δ T cells to 33%. The absolute number of γ/δ T cells, however, is reduced two-fold. Taken together, these data indicate that the deficiency of both LEF-1 and TCF-1 has a major impact on α/β T cell development, but does not appear to strongly affect the differentiation of cells into the γ/δ T cell lineage within fetal thymic organ culture.

Impairment of early T cell differentiation in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures.

With the aim of studying early stages of T cell differentiation in more detail, we analyzed the expression of CD25 and CD44 on DN T cells that were defined by the absence of the markers CD3, CD4, CD8, B220, TCRγ/δ, and NK1.1. Wild type, *Lef1*$^{-/-}$ *Tcf1(V)*$^{+/+}$ and *Lef1*$^{+/+}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures were found to contain similar proportions of cells representing the CD44^{-}CD25^{+} DN stage of T cell differentiation (Figure 3-3). In the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ thymic organ cultures, however, we observed a three-fold increase in the proportion of cells at this early developmental stage, suggesting that LEF-1 and TCF-1 control, at least in part, this step in T cell differentiation.

To explore the possibility that the incomplete block during the DN stage of differentiation was due to a defect in pre-TCR signaling, we experimentally induced the fetal thymic organ cultures with anti-CD3ε antibody [3]. Control organ cultures using thymuses from newborn RAG-1 deficient animals do not differentiate beyond the DN stage, but the addition of anti-CD3ε antibody (145-2C11), triggers maturation to the DP

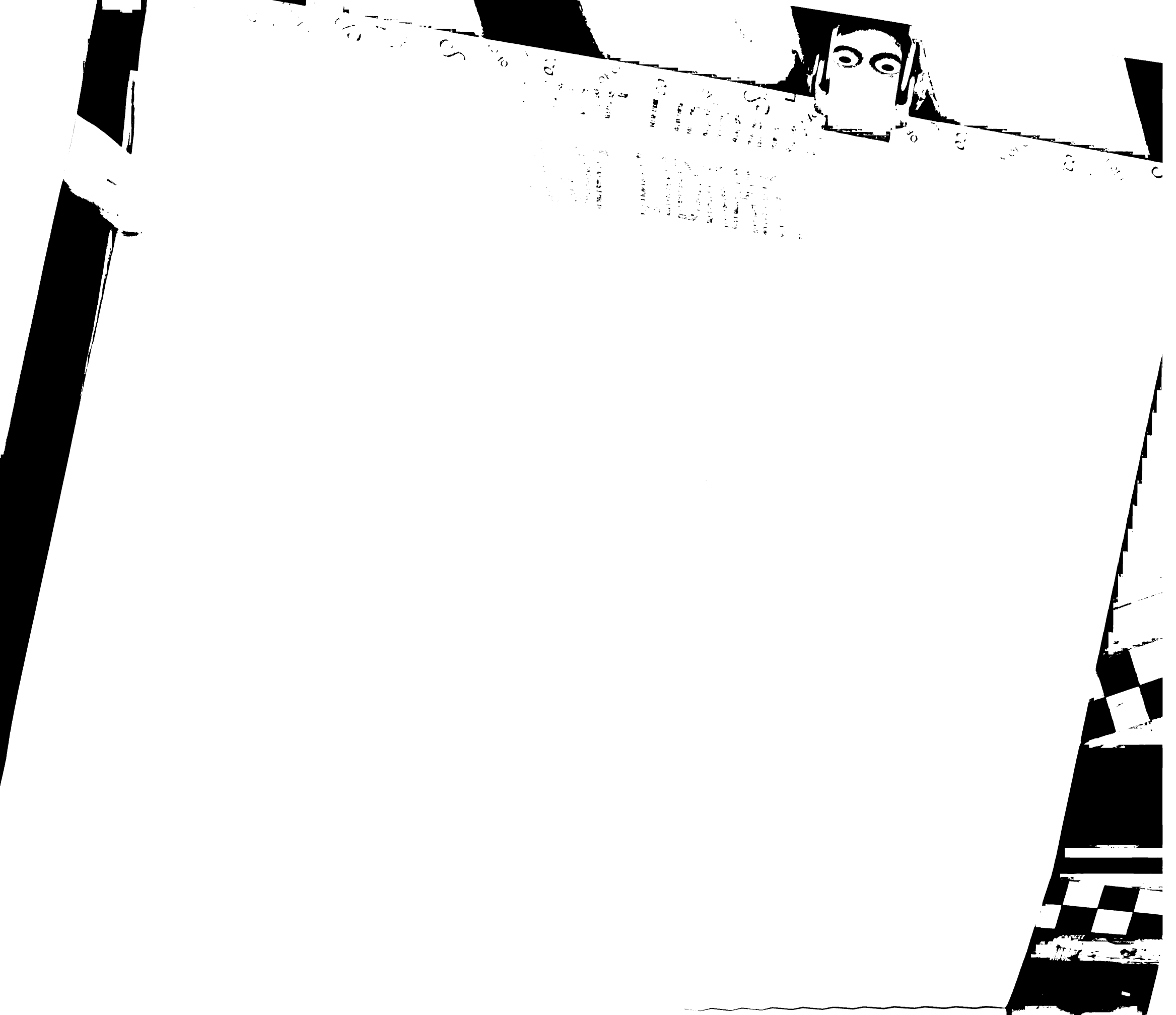
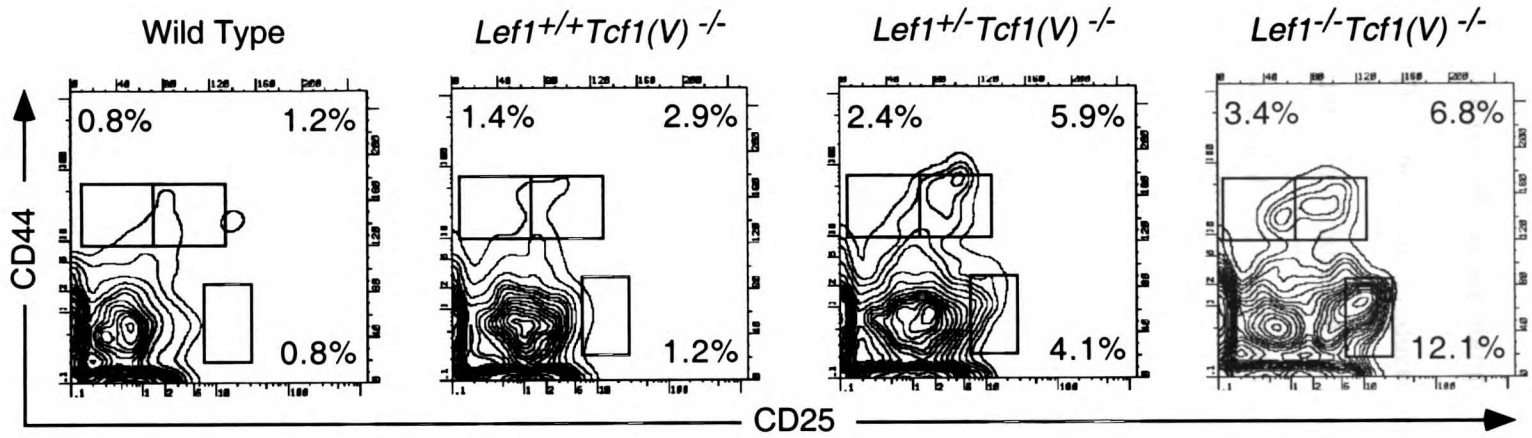


Figure 3-3. Incomplete block of T cell differentiation at the $CD44^{-}CD25^{+}$ DN stage in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures. Three-color flow cytometry analysis of E17.5 fetal thymic organ culture, after seven days of culture, using antibodies against mouse CD44, CD25. DN cells were gated for a lack of detectable surface expression of B220, CD3, CD4, CD8, NK1.1 and TCRγ/δ. The three DN populations ($CD44^{+}CD25^{-}$, $CD44^{+}CD25^{+}$, $CD44^{-}CD25^{+}$) are indicated by rectangles with the relative percentages of cells in each gate denoted. The total number of DN cells in each thymic organ culture is indicated above the contour plots.


Wild Type
Lef1+/+Tcf1(V) -/-
Lef1+/-Tcf1(V) -/-
Lef1-/-Tcf1(V) -/-
0.8%
1.2%
0.8%
1.4%
2.9%
1.2%
2.4%
5.9%
4.1%
3.4%
6.8%
12.1%
CD44
CD25

stage (Figure 3-4). *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ thymic organ cultures were also able to respond to the anti-CD3ε treatment by differentiating into DP thymocytes, suggesting that the signaling pathway used by the pre-T cell receptor is not abrogated in the double-deficient animals (Figure 3-4). In addition, CD4 expression is induced in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ thymic organ cultures by the antibody stimulation (Figure 3-4). Thus, CD4 transcription may not be dependent upon the expression of LEF-1 and TCF-1 despite the presence of a binding site in the murine CD4 enhancer [4].

LEF-1 and TCF-1 affect T cell differentiation in a T cell-autonomous manner.

To determine whether peripheral T cells develop from *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ precursors, we performed adoptive transfer experiments in immunodeficient SCID mice [5]. We injected $1x10^7$ E16.5 fetal liver cells intravenously into SCID mice and analyzed peripheral blood, spleen and thymus after ten weeks. In all adoptive transfers, B cells were detected in both blood and spleen at normal numbers confirming a successful reconstitution (Figure 3-5; Table 3-2). By contrast, only 2 out of 12 transfers of *Lef1*$^{+/-}$ *Tcf1*$^{-/-}$ fetal liver cells allowed for thymic reconstitution of mature $CD4^+$ and $CD8^+$ single positive T cells (Figure 3-5; Table 3-2). Notably, these two T cell reconstitutions did not generate any detectable peripheral T cells (Figure 3-5).

In adoptive transfers with fetal liver cells from *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ embryos, only 1 out of 12 yielded partial reconstitution of immature T lymphocytes within the thymus and none generated peripheral T cells (Figure 3-5; Table 3-2). Thus, the dependence of T cell differentiation on TCF-1 was more pronounced in the adoptive transfer assay than in fetal thymic organ cultures and the defect was further exacerbated by the absence of functional

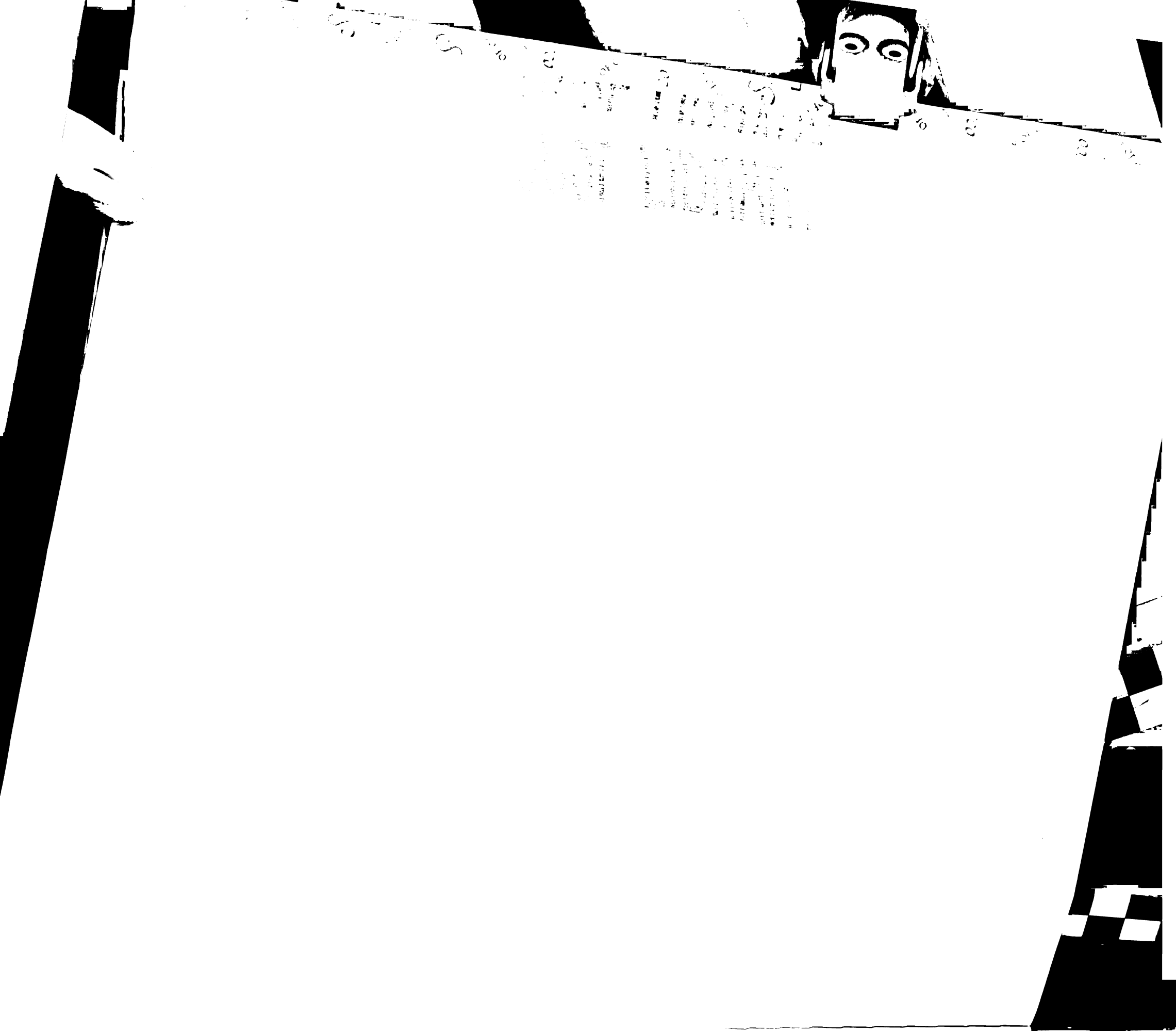
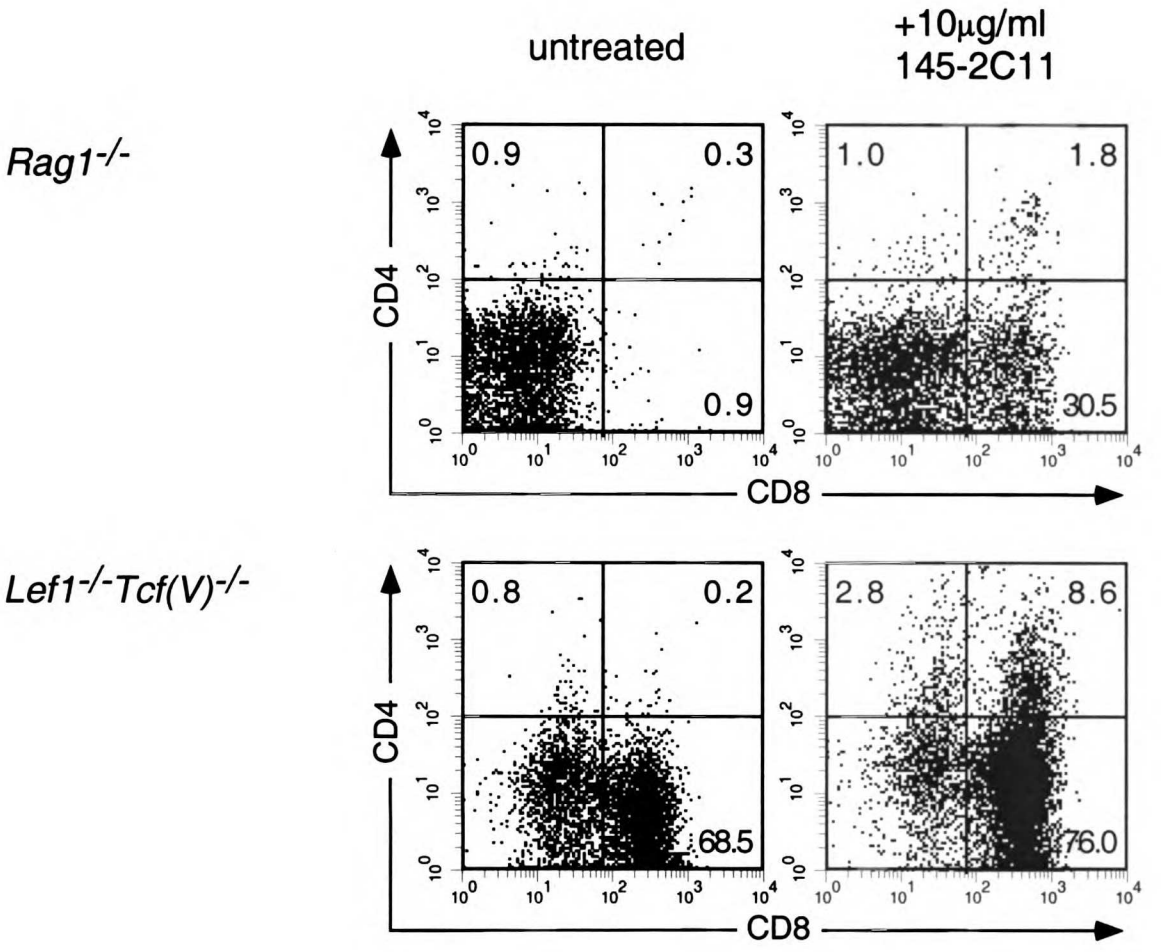


Figure 3-4. *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures retain the ability to mature in response to anti-CD3 antibody treatment. Two-color flow cytometry analysis of E17.5 fetal thymic organ culture, after five days of culture, with or without the addition of 10μg/ml of anti-CD3 antibody. Cells were stained using antibodies against mouse CD4 and CD8. Unstimulated wild type thymic organ cultures showed a similar profile as the wild type culture in Figure 3-1.


untreated
+10μg/ml
145-2C11
Rag1-/-
Lef1-/-Tcf(V)-/-
CD4
CD8
0.9
0.3
0.9
1.0
1.8
30.5
0.8
0.2
68.5
2.8
8.6
76.0

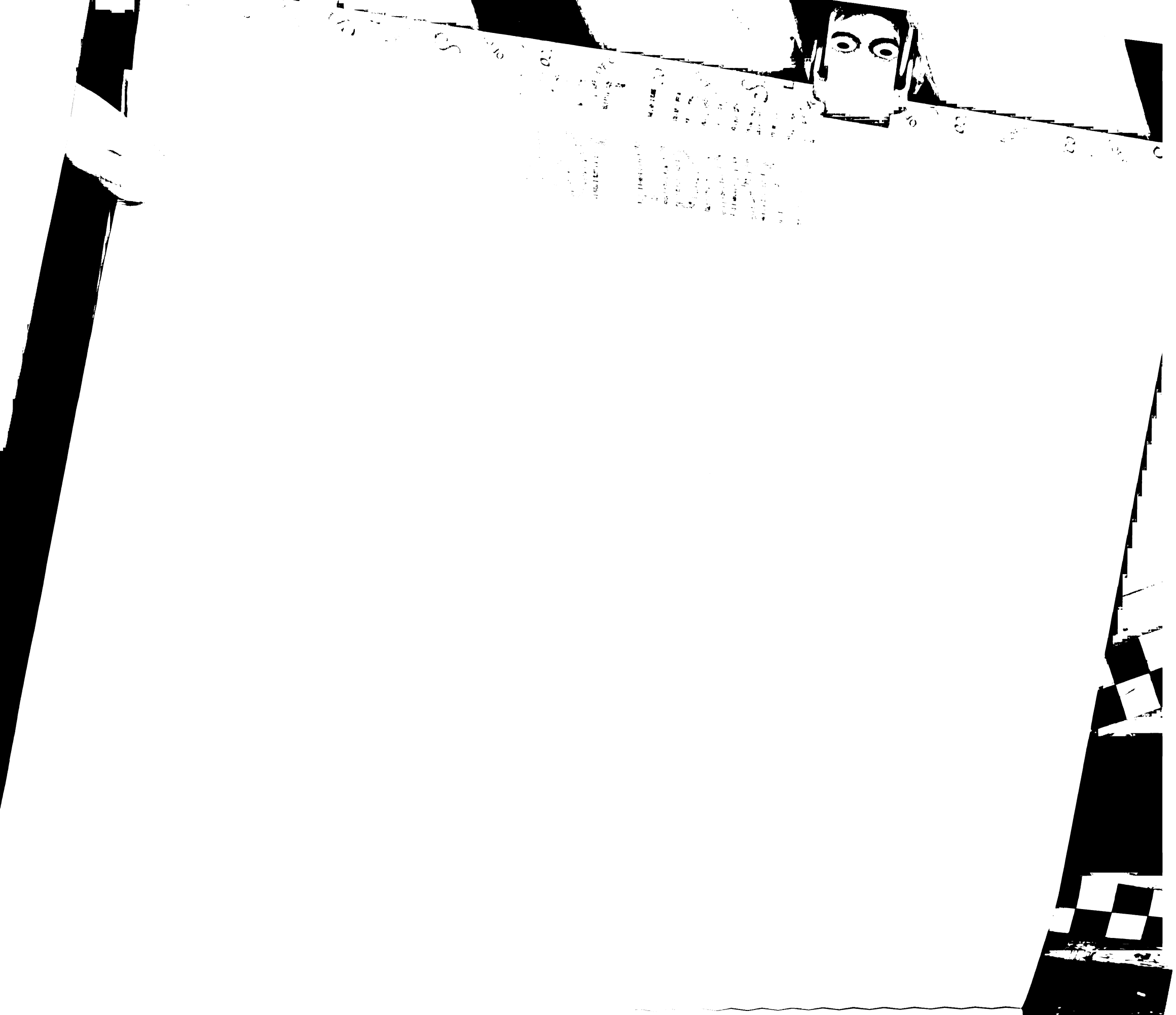


Figure 3-5. Adoptive transfer experiments using *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ E16.5 fetal liver display incomplete T cell development. (A) Two-color flow cytometry analysis of thymus derived from adoptive transfer experiments using antibodies against mouse CD4 and CD8. Data shown represents only successful T cell reconstitution of the total adoptive transfers using *Tcf1*$^{-/-}$ fetal liver precursors (*Lef1*$^{+/-}$*Tcf1*$^{-/-}$, 2/12, and *Lef1*$^{-/-}$*Tcf1*$^{-/-}$, 1/12; see table 2). (B) Two-color flow cytometry analysis of peripheral blood derived from adoptive transfer experiments using antibodies against mouse CD4, CD8, IgD_b, and IgM_b. Tissues were removed 10 weeks after I.V. transfer of $1x10^7$ fetal liver cells. Peripheral blood was collected via tail bleed and red cells were lysed with 150mM NH_4CL, 10mM $KHCO_3$, and 0.1 mM EDTA.

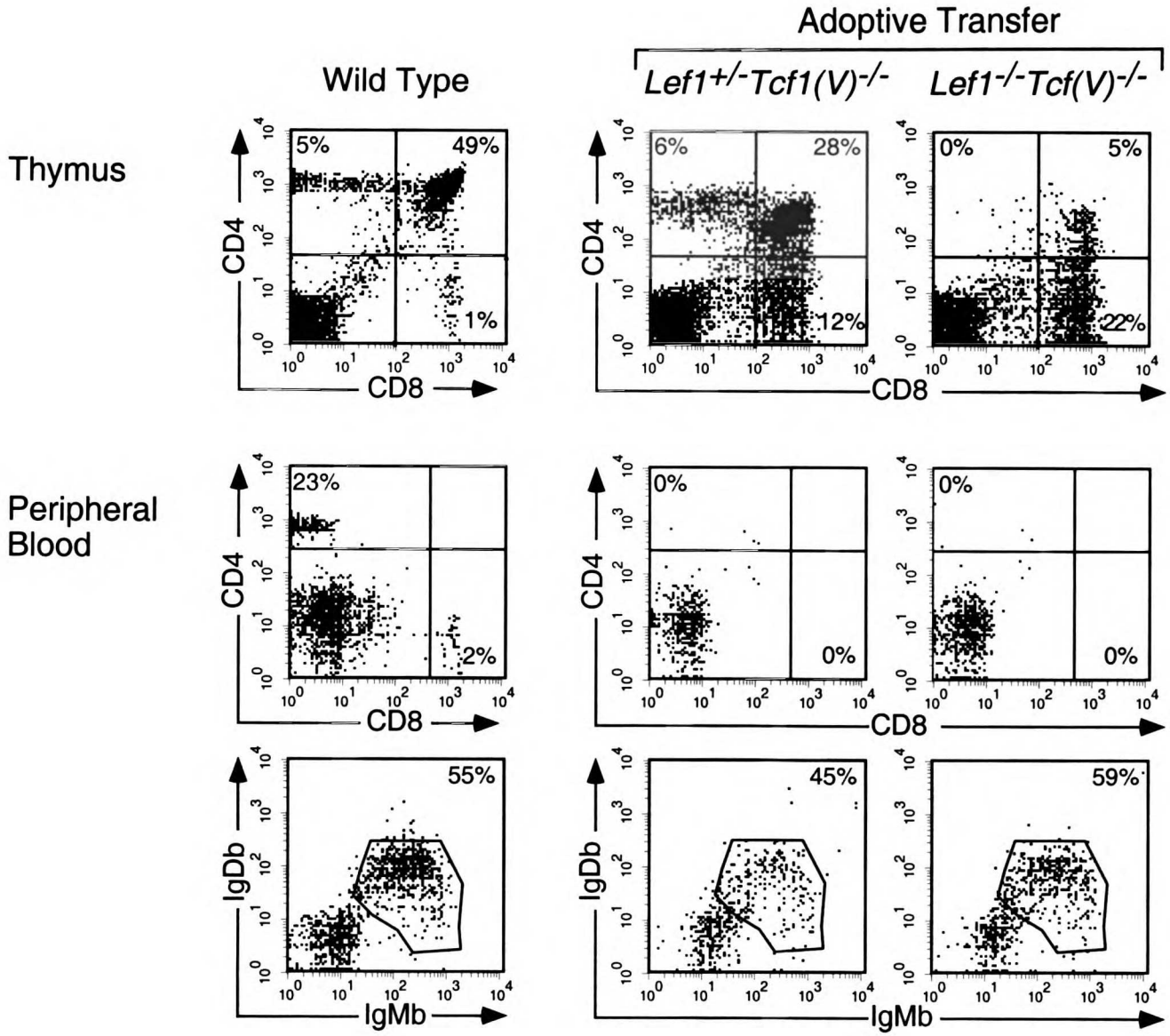
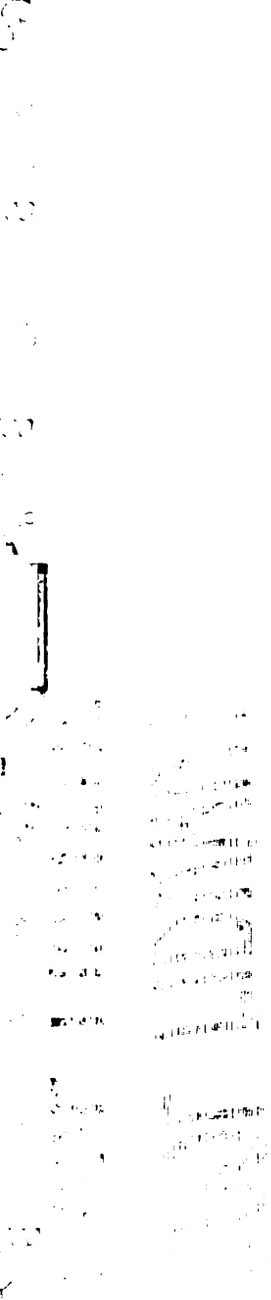

Adoptive Transfer
Wild Type
Lef1+/-Tcf1(V)-/-
Lef1-/-Tcf(V)-/-
Thymus
Peripheral Blood
CD4
CD8
IgDb
IgMb
5%
49%
1%
6%
28%
12%
0%
5%
22%
23%
2%
0%
0%
0%
0%
55%
45%
59%

Table 3-2. Frequency of lymphocyte recovery in adoptive transfer experiments

			thymus	spleen	
donor	recipient	# of transfers	T cells	T cells	B cells
no donor	BALB/c SCID	2	0/2	0/2	0/2
C57/bl6	BALB/c SCID	18	18/18	18/18	18/18
Lef1$^{-/-}$*Tcf1*$^{+/+}$	BALB/c SCID	36	36/36	36/36	36/36
Lef1$^{+/-}$*Tcf1(V)*$^{-/-}$	BALB/c SCID	12	2/12	0/12	12/12
Lef1$^{-/-}$*Tcf1(V)*$^{-/-}$	BALB/c SCID	12	1/12	0/12	12/12

All experiments were analyzed 12 weeks after intravenous transfer of $1x10^7$ E16.5 fetal liver cells into BALB/c SCID mice. Presence of T cells and B cells within the thymus and spleen was detected by FACS, marking T cells with antibodies against CD4 and CD8 and B cells with allotype specific antibodies against IgD and IgM. Adoptive transfers which contained lymphocytes that amounted to at least 5% of the total tissue were scored as successful reconstitutions.



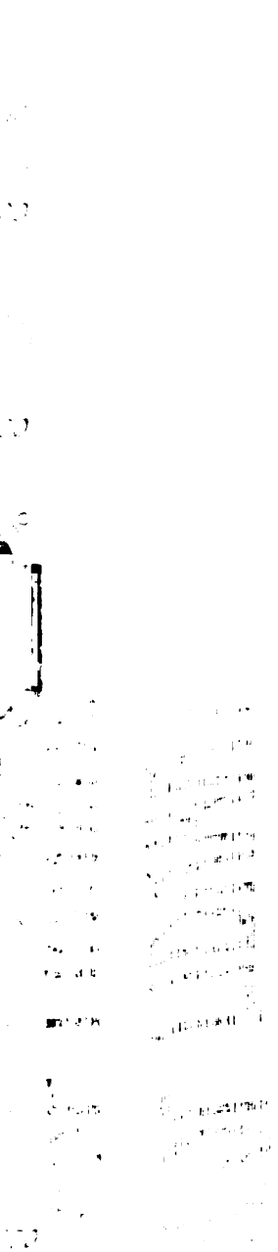
LEF-1 protein. In addition, the phenotype of T cell differentiation in the single successful reconstitution using the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal liver precursors resembled that of the fetal thymic organ cultures. These data demonstrates that the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ T cell differentiation defects are T cell-autonomous and are not caused by the double-deficient thymic stroma.

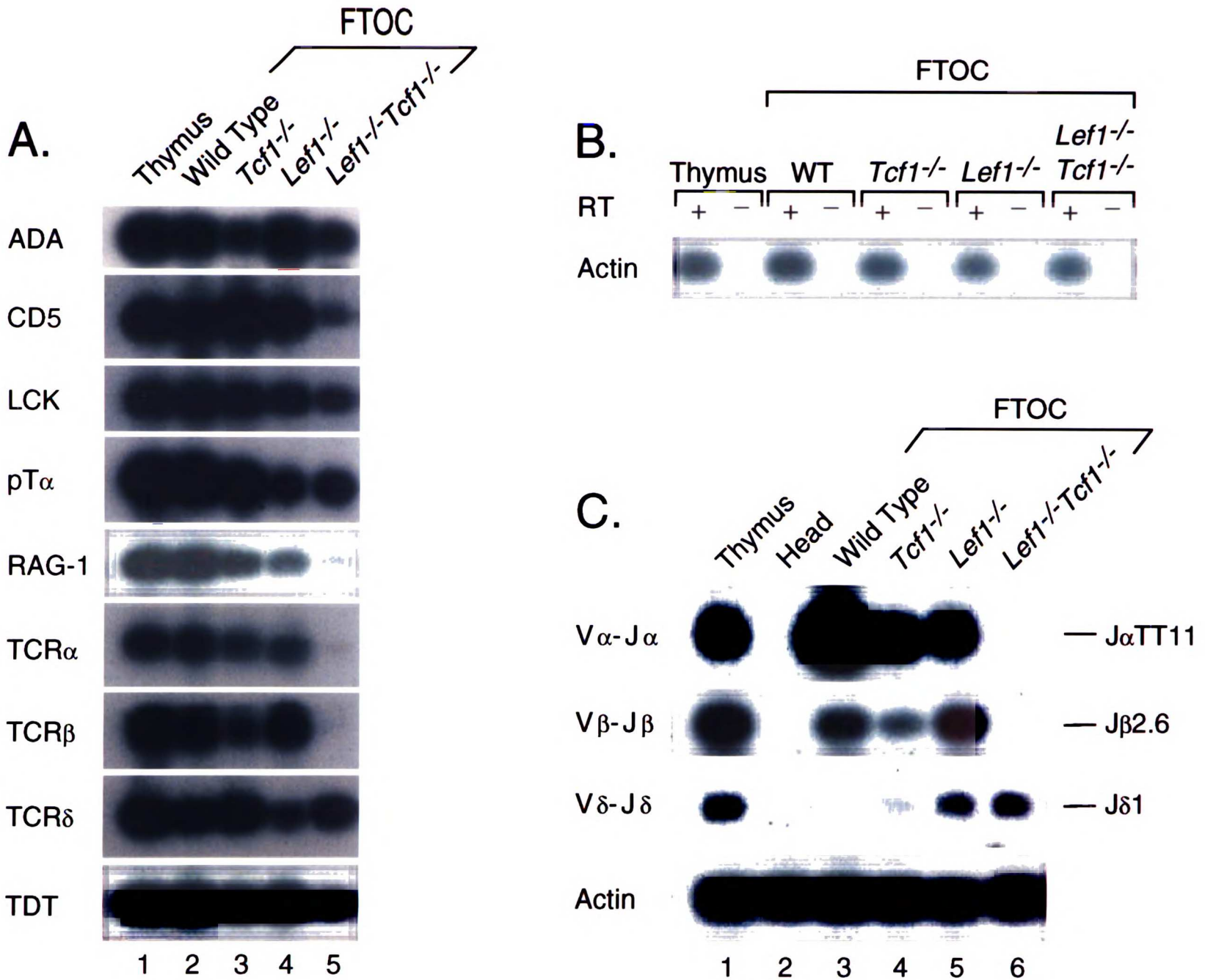
Expression and rearrangement of the TCRα locus is severely impaired in *Lef1*$^{-/-}$ *Tcf1*$^{-/-}$ thymocytes.

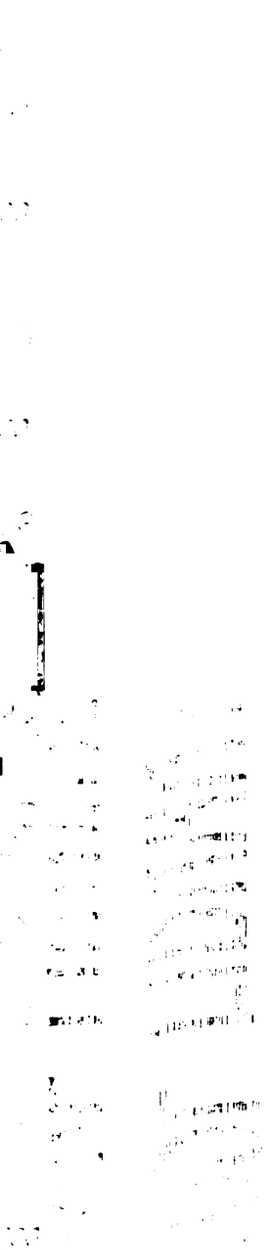
Rearrangement and expression of the various TCR loci defines important regulatory steps in T cell differentiation. To gain further insight into the molecular defects in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures, we analyzed the mRNA for the presence of transcripts from the TCR loci and from genes that are expressed during earlier T lymphocyte differentiation, such as ADA, CD5, LCK, pTα, RAG-1 and TdT. Amplification of cDNA from multiple thymic organ cultures by PCR and visualization of the products by hybridization with specific DNA probes indicated that *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ thymocytes generate transcripts from the ADA, CD5, LCK, pTα, RAG-1, TCRδ and TdT genes but contain reduced levels of TCRα and TCRβ mRNA (Figure 3-6A and B). By contrast, expression of the TCRα and TCRβ genes was detected at normal levels in fetal thymic organ cultures from both *Lef1*$^{-/-}$ and *Tcf1*$^{-/-}$ single gene disruptions.

Expression of the TCRα and TCRβ loci is preceded by rearrangement of the V, D and J gene segments. Rearrangement of the TCRβ, TCRγ, and TCRδ loci occurs predominantly in CD44^{-}CD25^{+} DN stage of T cell differentiation, whereas rearrangement of the TCRα occurs during the CD4^{+}CD8^{+} double positive stage [6]. The rearrangement

Figure 3-6. Analysis of gene expression and TCR gene rearrangements in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ fetal thymic organ cultures. (A) Expression of the ADA, CD5, LCK, pTα, TCRα, TCRβ, TCRδ, TdT, and RAG-1 genes in fetal thymic organ cultures (FTOC). mRNA was isolated from neonatal thymus or from E17.5 fetal thymic organ cultures after seven days of culture. cDNA was generated by reverse transcription (RT), amplified by PCR and the products were separated by gel electrophoresis and visualized by hybridizing DNA blots with labeled probes. The genotypes of the fetal thymic organ cultures were determined by DNA blot analysis and are indicated above the autoradiograms of the cDNA products. (B) Expression of actin was used as a control for the quality of mRNA and as an estimate of the quantity of cDNA used in each lane. To control for a potential contamination of the mRNA preparations by genomic DNA, PCR reactions were also performed in the absence of reverse transcription. (C) Analysis of TCRα, TCRβ and TCRδ gene rearrangements in total fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice. PCR was performed using genomic DNA isolated from neonatal thymus, head or E17.5 fetal thymic organ cultures after seven days of culture. DNA blots of PCR products were hybridized with radiolabeled oligonucleotides specific for actin or for specific TCRα, TCRβ and TCRδ recombination products. Each lane represents an independent organ culture. Long exposures of the TCRβ blot revealed a low level of rearrangement whereas similar exposures of the TCRα blot did not reveal any detactable rearrangements.



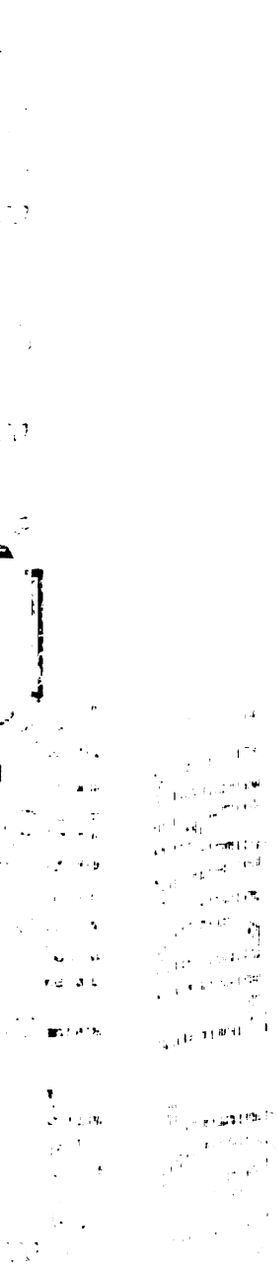

A.
FTOC
Thymus
Wild Type
Tcf1-/-
Lef1-/-
Lef1-/-Tcf1-/-
ADA
CD5
LCK
pTα
RAG-1
TCRα
TCRβ
TCRδ
TDT
1 2 3 4 5
B.
FTOC
Thymus
WT
Tcf1-/-
Lef1-/-
Lef1-/-
Tcf1-/-
RT
+ − + − + − + − + −
Actin
C.
FTOC
Thymus
Head
Wild Type
Tcf1-/-
Lef1-/-
Lef1-/-Tcf1-/-
Vα-Jα
— JαTT11
Vβ-Jβ
— Jβ2.6
Vδ-Jδ
— Jδ1
Actin
1 2 3 4 5 6

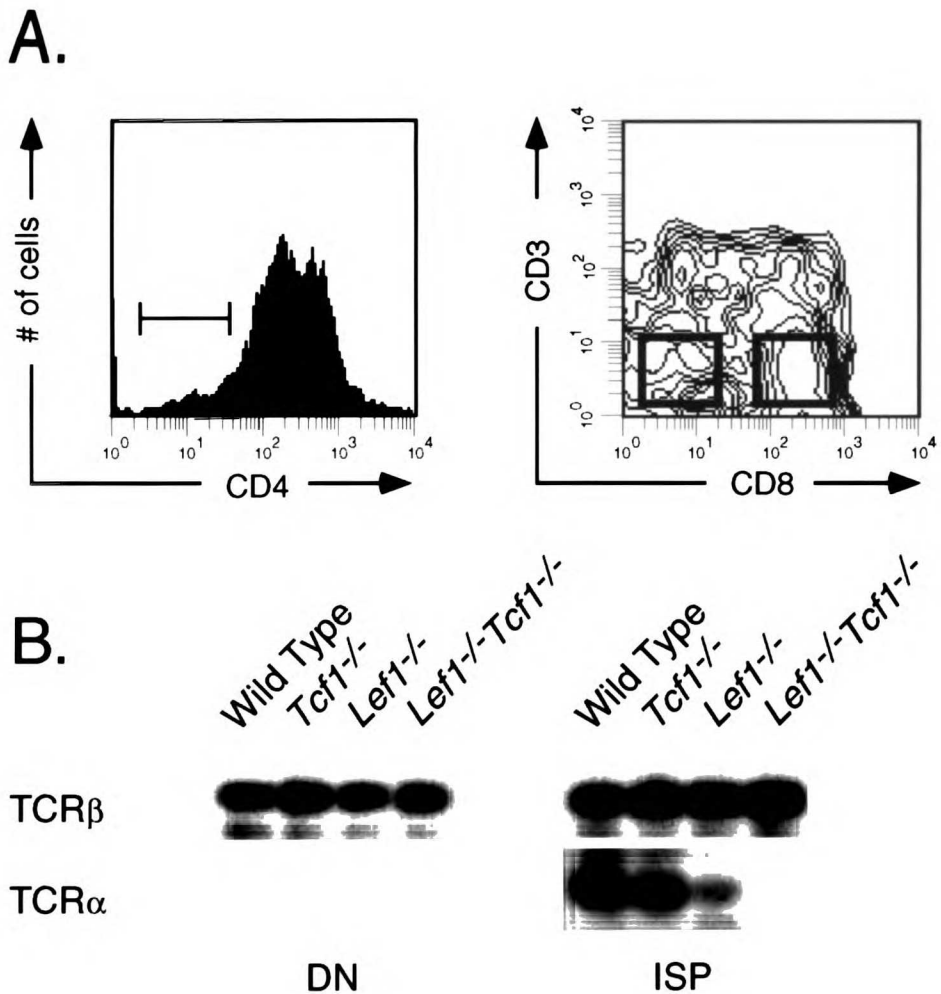


status of the TCR loci was examined by PCR analysis of genomic DNA from fetal thymic organ cultures and by probing the DNA blots with TCRα, TCRβ and TCRδ DNA fragments (Figure 3-6C). Abundant V(D)J recombination was observed for both TCRα and TCRβ loci in LEF-1 and in TCF-1 deficient thymocytes. However, no rearrangements of Vα to Jα at the TCRα locus were detected in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures and only a low level of V(D)J recombinations at the TCRβ locus could be visualized on long exposures of the autoradiogram (data not shown). Abundant TCRδ locus recombination was detected in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures demonstrating that V(D)J recombination can occur in the absence of LEF-1 and TCF-1 (Figure 3-6C).

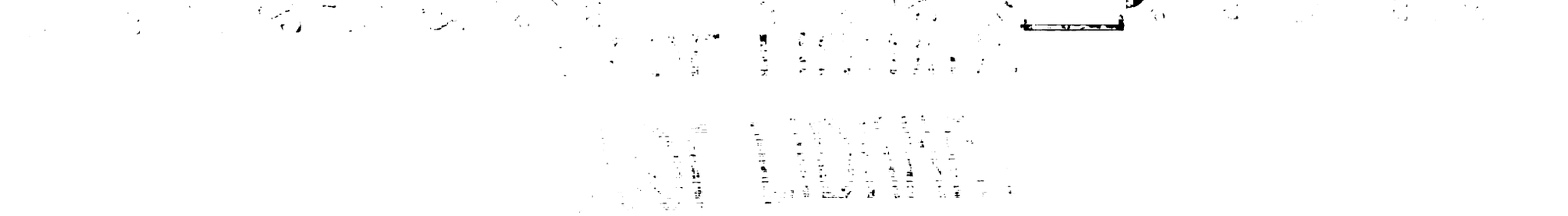
The relative low abundance of transcripts from the TCRα and TCRβ genes in the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures could be due to either the absence of the DP and mature CD4$^+$ and CD8$^+$ single positive (MSP) T cell populations, or alternatively, due to the absence of gene expression. To distinguish between these possibilities, we repeated our mRNA analysis with sorted DN and CD8$^+$ ISP cells from fetal thymic organ cultures of wild type, *Lef1*$^{-/-}$, *Tcf1(V)*$^{-/-}$ and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ embryos (Figure 3-7). As anticipated, TCRα mRNA is undetectable in the DN fraction of all organ cultures and is present in the CD8$^+$ ISP fraction of the wild type, *Lef1*$^{-/-}$, and *Tcf1(V)*$^{-/-}$ organ cultures. However, TCRα mRNA is greatly reduced in the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ CD8$^+$ ISP fraction, consistent with the result obtained with the unfractionated thymus mRNA. In contrast, TCRβ is expressed at similar levels in the cells of the DN and CD8$^+$ ISP T cells of all tested genotypes, including the homozygous double mutant mice. All DN and CD8$^+$ ISP fractions tested contained equivalent amounts of TCRβ mRNA. Thus, the decrease in

Figure 3-7. Analysis of TCRα and TCRβ mRNA expression in DN and CD8+ ISP T cells. (A) Flow cytometry of thymocytes from E17.5 fetal thymic organ cultures that have been cultured for seven days. Cells were sorted for lack of surface expression of CD4 (left panel) and CD3 (right panel). Cells that met these criteria were further sorted for expression of CD8. $CD3^-CD4^-CD8^-$ cells (right panel, left gate) were used as DN T cells and $CD3^-CD4^-CD8^+$ cells (right panel, right gate) were used as $CD8^+$ ISP T cells. (B) RT-PCR analysis of mRNA from sorted DN and $CD8^+$ ISP T cells. cDNA was prepared from sorted cell populations of the various genotypes and amplified by PCR with primers for either TCRα and TCRβ. The RT-PCR products were visualized by DNA blot analysis and hybridization with probes for constant region sequences from the TCRα and TCRβ loci.




A.
# of cells
CD4
CD3
CD8
B.
Wild Type
Tcf1-/-
Lef1-/-
Lef1-/-Tcf1-/-
TCRβ
TCRα
DN
ISP

TCRβ transcription detected in the unfractioned *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures is likely due to the marked reduction in the absolute number of T cells and the absence of DP and MSP T cells that normally express TCRα and TCRβ at high levels. The absence of cells representing the latter stages of T cell differentiation may also account for the modest decrease in the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures in the levels of CD5 and RAG-1 expression which are upregulated in DP T cells.



DISCUSSION

In this study we show that the closely related transcription factors LEF-1 and TCF-1 regulate T cell differentiation in a partially redundant manner. T cell differentiation is severely impaired, in fetal thymic organ cultures from mice carrying mutations in both *Lef1* and *Tcf1* genes, but not in cultures from mice carrying single mutations in either gene alone. Specifically, we show that at least two steps in the differentiation of α/β T cells are affected in fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. The differentiation of T cells from CD8^{+} ISP cells to CD4^{+}CD8^{+} DP cells is completely arrested. Moreover, an incomplete block at the CD44^{-}CD25^{+} DN stage of development is observed. In contrast, differentiation of γ/δ T cells in the thymus appears to be largely unaffected, suggesting that LEF-1 and TCF-1 specifically regulate differentiation of the α/β T cell lineage.

The observed defects in T cell differentiation in the double mutant mice cannot be accounted for by the loss of expression of known presumed target genes for LEF-1 and TCF-1. The incomplete block at the CD44^{-}CD25^{+} DN stage of T cell differentiation is similar to the arrest of T cell differentiation seen in mice deficient in components of the pre-T cell signaling pathway. Mice carrying targeted gene disruptions in the TCRβ locus [7] have a complete block in differentiation at this stage, however our analysis suggests that TCRβ transcription is largely unaffected by the absence of functional LEF-1 and TCF-1. A potential binding site for LEF-1 and TCF-1 has been identified in the human TCRβ enhancer [8], but the functional analysis of both the human and the murine TCRβ enhancer did not include this transcription factor binding site [9, 10]. An incomplete arrest of T cell differentiation at the CD44^{-}CD25^{+} DN stage has also been reported in

mice carrying mutations in the *lck* gene [11]. Although, the promoter of this gene contains a site that is recognized by LEF-1/TCF-1 *in vitro* [12], the *lck* gene is expressed at normal levels in fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. Finally, mutation of the pTα gene results in an incomplete block at the CD44^{-}CD25^{+} DN stage [13]. This gene, however, is also expressed at normal levels in fetal thymic organ cultures from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. Although we cannot rule out that another component of the pre-T cell receptor signaling pathway is dependent on the expression of LEF-1 and TCF-1, our experiment in which we used an anti-CD3 antibody to artificially stimulate differentiation suggests that this pathway may be, at least partially, intact.

Another potential target gene for regulation by LEF-1 and TCF-1 is the *Ada* gene encoding the enzyme adenosine deaminase, which is required for the proper maturation of developing thymocytes. Studies of the human ADA enhancer have identified a LEF-1/TCF-1 binding site which is important for the insertion-site independent expression of transgenes in the thymus of transgenic mice [14]. However, the *Ada* gene is expressed in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures at apparently normal levels. Thus, none of these endogenous genes that contain biochemically identified LEF-1/TCF-1 binding sites in transcriptional control regions are critically dependent upon these transcription factors *in vivo*.

The minimal TCRα enhancer has been shown to be regulated by LEF-1, and to a lesser extent by TCF-1, in transient transfection assays [15-18]. Although the LEF-1/TCF-1 binding sites appear to be dispensable for the activity of a larger TCFα enhancer fragment in transient transfection assays [19], we found a marked reduction of the expression and rearrangement of the endogenous TCRα locus in thymic organ cultures

from *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. In addition, our analysis of sorted CD8^{+} ISP T cells revealed that transcription of the constant region of the TCRα locus is greatly reduced in the *Lef1*$^{-/-}$ *Tcf1*$^{-/-}$ mice but unaffected in either *Lef1*$^{-/-}$ or *Tcf1*$^{-/-}$ mice. The marked decrease in the expression of the TCRα locus in sorted CD8^{+} ISP T cells, which are present in the *Lef1*$^{-/-}$ *Tcf1*$^{-/-}$ mice and contain normal levels of TCRβ transcripts, indicates that LEF-1 and TCF-1 directly control the expression of the TCRα locus in a redundant manner. These findings also suggest that the locus control region, which has been identified in the 3' region of the TCRα locus [20], either fails to compensate for the functional deficiency of the TCRα enhancer or involves regulation by LEF-1 and TCF-1.

The complete arrest of differentiation at the immature CD8^{+} single positive stage in the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ thymocytes cannot yet be accounted for by a change in the expression of known presumed target genes. However, treatment of fetal thymic organ cultures with an antibody against murine CD81 appears to produce defects in T cell differentiation similar to those observed in *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures [21]. Organ cultures treated with anti-CD81 antibodies failed to produce double positive cells but generated abundant numbers of immature CD8^{+} single positive cells. In addition, a second block in T cell differentiation at the CD25^{+} double negative stage was described. The authors attribute the block in development to the prevention of an essential interaction between stromal-expressed CD81 and an unknown receptor on the surface of the developing lymphocyte. Given that the adoptive transfer experiments with *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ precursors demonstrated a similar defect to the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ culture, it is not likely that a deficiency of stromal CD81 is responsible, but it remains possible that the receptor for CD81 may be a target of LEF-1 and TCF-1.

In comparison with the analysis of adult *Tcf1*$^{-/-}$ mice which revealed an incomplete block at the CD8^{+} ISP stage [2, 22], the fetal thymic organ cultures from the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice show a full arrest of differentiation at the same developmental stage and, in addition, a more pronounced block at the earlier CD44^{-}CD25^{+} DN stage. Although adoptive transfer experiments indicated similar qualitative defects in the appearance of the different T cell populations within the thymus, these experiments also revealed a pronounced dependence of T cell reconstitution on TCF-1. This deficiency in reconstitution of T cells could be explained, in principle, by the previously reported defect in the number of actively dividing cells in the thymus of adult *Tcf1*$^{-/-}$ mice [2].

Several mechanisms may account for the differential phenotype of the double mutant and single mutant mice. First, LEF-1 and TCF-1 may be functionally redundant. However, a simple redundancy between these transcription factors is unlikely because *Tcf1*$^{-/-}$ mice already display a phenotype that is further augmented by a mutation in the *Lef1* gene. This contrasts with the redundancy observed in the regulation of other cell lineages. For example, single targeted gene inactivations of the myogenic transcription factors genes *MyoD* and *Myf-5* fail to produce an obvious defect in muscle differentiation [23, 24], whereas mice carrying mutations in both genes display a severe defect in muscle formation [25] (reviewed in Lassar and Munsterburg, 1994). The phenotype of the *Lef1*$^{-/-}$ *Tcf1*$^{-/-}$ mice could be formally explained by the difference in the relative abundance of redundant transcription factors. Consistent with this scheme, TCF-1 is significantly more abundant than LEF-1 in the thymus [18]. Thus, the absence of the abundant transcription factor TCF-1, but not the absence of the less abundant factor LEF-1, may result in a

partial phenotype in thymic development which is further impaired by a lack of both factors.

A second mechanism could involve a functional synergy between LEF-1 and TCF-1 whereby the two transcription factors would regulate distinct cellular processes, which, together, are required for normal T cell differentiation. According to this view, LEF-1 and TCF-1 would regulate separate sets of target genes. Although the two transcription factors can bind the same sites *in vitro* and display sequence homology outside the HMG domain, both proteins also contain distinct domains that account for differential transcriptional activation potentials. For example, LEF-1 contains a context-dependent transcriptional activation domain [26, 27] that interacts with a ubiquitously expressed nuclear protein, ALY [28]. In addition to association with LEF-1, ALY also interacts with the transcription factor AML-1, which recognizes two sites adjacent to the LEF-1 binding site in the TCRα enhancer [28]. ALY is required for TCRα enhancer function and it interacts with LEF-1 more efficiently than with TCF-1 [28] which may account for the more efficient activation of the minimal TCRα enhancer by LEF-1 [18].

Recently, studies have shown that LEF-1 and TCF-1, in association with β-catenin can mediate transcriptional activation independent of a specific context of other transcriptional factor binding sites [29]. However, this association of LEF-1/TCF-1 with β-catenin may not be important for the regulation of the TCRα enhancer because β-catenin does not affect LEF-1-mediated transcriptional stimulation of the TCRα enhancer [30]. Moreover, the HMG domain of LEF-1 lacking the N-terminal β-catenin interaction domain, and truncated isoforms of TCF-1 stimulate the activity of the TCRα enhancer [17, 18]. The role of Wnt signaling in thymic T cell differentiation remains unclear but

β-catenin:LEF-1/TCF-1 complexes may be responsible for regulating some of the phenotypes observed in the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ fetal thymic organ cultures.

In conclusion, the transcription factors LEF-1 and TCF-1 play an important and, at least partially, redundant role in T cell differentiation and in the regulation of the TCRα locus. Although the TCRα gene is a likely target for these transcription factors, the absence of TCRα expression cannot account for the entire T cell phenotype of the *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ mice. The identification of other functionally important genetic targets of these transcription factors may provide insight into the regulatory network governing the transcriptional control of T cell differentiation.

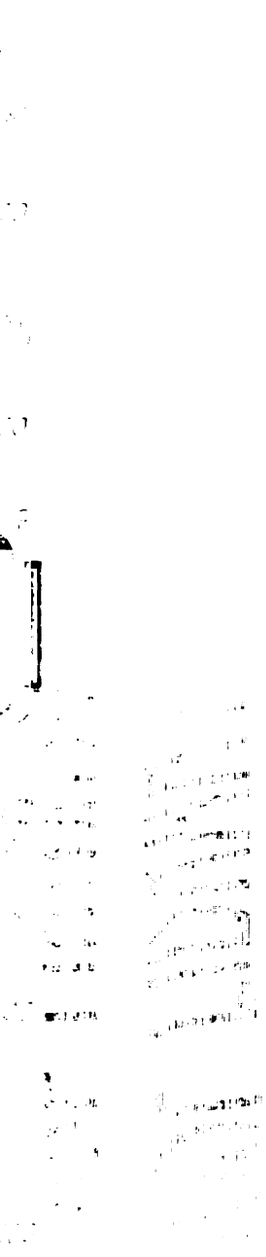
Materials and methods

Mice

C57BL/6 and BALB/c SCID mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained in the Animal Care Facility at the University of California, San Francisco (UCSF). LEF-1 and TCF-1 deficient mice were generated as previously described (van Genderen et al., 1994; Verbeek et al., 1995). All experimental adult animals were between the ages of 8-12 weeks of age.

Fetal thymic organ cultures

Thymi were isolated from embryos at E17.5. Both lobes were placed on autoclaved polycarbonate membranes (Costar) suspended by metal grids over the inner well of Falcon 3037 tissue culture plates (Falcon). RPMI supplemented with 10% fetal calf serum, sodium pyruvate, penicillin/streptomycin, non-essential amino acids, vitamins, and 2-mercaptoethanol was used as culture medium in the inner well, maintaining contact with the bottom surface of the membrane only. PBS was used in the outer well to maintain a humid environment. Organ cultures were kept at 37°C with 5.0% CO_2 for seven days. Medium was replaced each day during this period. After culture, lobes were dispersed and cells were filtered over nylon membrane. Stimulation of organ cultures with anti-CD3ε antibody (145-2C11, 10μg/ml) was performed by adding antibody at the start of culture and analysis of antibody treated and untreated cultures was performed after five days of culture. For the anti-CD3ε experiments, the media was not changed during the course of culture.



Adoptive transfers

Fetal liver was isolated from E16.5 embryos and dispersed by standard methods. The resultant suspension was then filtered over nylon and cells were resuspended at $1x10^8$/ml in RPMI + 10%FCS. BALB/c SCID mice (Jackson Laboratories) were used as recipients in all experiments. $1x10^7$ fetal liver cells were injected I.V. into the tail vein of each mouse and transfers were sacrificed after ten weeks.

Flow cytometry

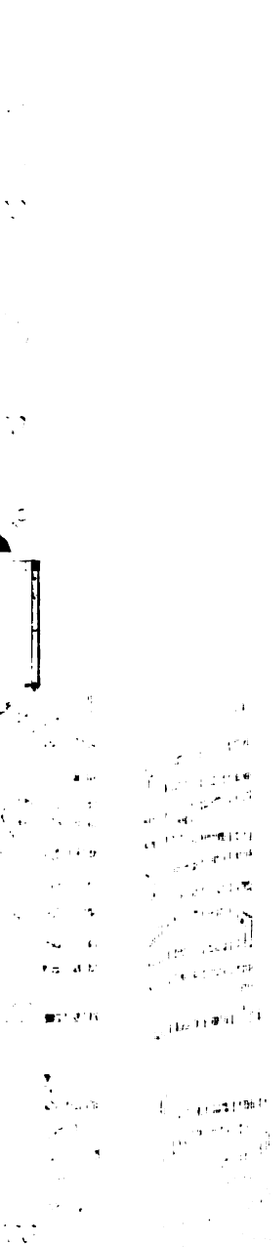
$1x10^7$ cells were resuspended in 25µl staining medium (PBS + .3% BSA) and incubated with antibody for 15'. Cells were then washed three times with staining medium and the process was then repeated with secondary antibodies as necessary. After staining, cells were resuspended in 400µl staining medium with 200ng/ml propidium iodine. Cells were then analyzed using either a FACStar plus (Becton Dickinson), a FACSCalibur (Becton Dickinson) or a modified, dual laser FACS II (FLASHER, Stanford Shared FACS Facility, Stanford). Data generated was interpreted using Cellquest (Becton Dickinson) for experiments performed on the FACStar plus and the FACSCalibur or using FACS-DESK(Stanford Shared FACS Facility, Stanford) for data collected on FLASHER. Data used in figures is gated for lymphocytes by size and complexity and for live cells by excluding cells that uptake propidium iodine. Plots display 5% probability or dot plot representation.

Antibodies

Directly conjugated antibodies specific for mouse B220, CD3ε, CD4, CD8, TCRβ, and TCRγ/δ were obtained from Caltag (San Francisco, California). Directly conjugated antibodies specific for mouse CD25, CD44, IgD_b, IgM_b, NK1.1, Thy1.2 and streptavidin-conjugated allophycoerythrin were obtained from Pharmingen (La Jolla, California).

RNA and DNA isolation and analysis

Total mRNA and DNA was obtained from whole fetal thymic organ cultures using TRIzol (BRL) and mRNA from sorted cells was generated by using an mRNA capture kit (Boehringer Mannheim); both products were used according to manufacturers recommendations with no modifications. Half of the mRNA was used for cDNA production with 200nM random hexamers (Pharmacia) and reverse transcriptase (Boehringer Mannheim) in commercial buffer (Boehringer Mannheim) modified by the addition of 3U Rnasin. Reactions were incubated for 60' at 37°C. rtPCR amplification of cDNA was performed in 10μl total volume with 1μM primers, 0.2 mM dNTP, 0.5U Taq polymerase (Boehringer Mannheim) in Taq polymerase buffer. All rtPCR reactions, except for the ADA rtPCR, were normalized for actin and were done under the following amplification conditions: 45" at 94°C, 45" at 61°C, and 1' at 72°C. ADA rtPCR was modified to 45" at 94C, 45 at 57°C, and 1' at 72°C. Actin rtPCR was performed for 30 cycles and all other rtPCR used 35 cycles. Genomic PCR was performed under identical reaction conditions and used the following amplification protocol: 75" at 94°C, 75" at 62°C, and 90" at 72°C, for 30 cycles. PCR products were transferred to Hybond N+ (Amersham) nylon supported nitrocellulose for 8-12 hours. Prehybridization/hybridization was done at 65°C O/N in 6x SSC, 5x Denharts, 0.1%



SDS, 0.5 mg/ml salmon sperm DNA. RAG-1, CD5, TdT and actin were hybridized with random primed cDNA and all other probes used were end-labeled oligonucleotides. Oligonucleotides used for rtPCR and generation of probes are as follows: actin sense, GTTTGAGACCTTCAACACC; actin anti-sense GTGGCCATCCCTGCTCGAAGTC; ADA (probe), GAGACCATTGAAGGAAGTAGCCTC; ADA sense, GAAGTACAATCAGGAGACCCGTGG; ADA anti-sense, CTGTTGTAGAGAGCTTCATCCTCG; CD5 sense, TTCTGCCTCGGACAGTCTGG; CD5 anti-sense, GCCTGTCCTTGGCCTTGTAG; LCK (probe), GCAGCTGCTTCATGAGGTTAGC; LCK sense, GGTCAGAGACTTCGACCAGAAC; LCK anti-sense, CCACTGCATAAAGCCGGACTAG; pTα (probe), AGTTTGAAGAGGAGCAGGCGCAG; pTα sense, CACACTGCTGGTAGATGGAAGGC; pTα anti-sense, GTCAGGAGCACATCGAGCAGAAG; RAG-1 sense, TGCAGACATTCTAGCACTCTGG; RAG-1 anti-sense, ACATCTGCCTTCACGTCGAT; TCRα (probe), TTCTCAGTCAACGTGGCATCACA; TCRα sense, CCAGAACCTGCTGTGTACCAGTT; TCRα anti-sense, ACAGCCTCAGCGTCATGAGCAG; TCRβ (probe), TTCAGAGGAGGACAAGTGGC; TCRβ sense, AAAGGCTACCCTCGTGTGC; TCRβ anti-sense, GGATGGTTGCAGACAGAACC; TCRδ (probe), ACCCTAAAGAGGTGACTATAAGTC; TCRδ sense, GGAACAAATGTTGCTTGTCTGGTG; TCRδ anti-sense, AATGGTCTTGGCAAACAGCAGTCG; TdT sense,

GAAGATGGGAACAACTCGAAGAG; TdT anti-sense,

GACGTGCTGGAACATTCTGGGAG. Oligos used for TCR gene rearrangement assays are as follows (Levin et al., 1993; Carroll and Bosma, 1991): JαTT11 (probe),

GAAAGCAGAGTCCCAATTCCAAAG; JαTT11-3',

GACCCTATTACTCACATACTTGGCTTG; Vα2C-5',

ACTGTCTCTGAAGGAGCCTCTCTG; VαF3-5',

ACCCAGACAGAAGGCCTGGTCACT; VαH-5',

CAGAAGGTGCAGCAGAGCCCAGAA; JB2 (probe),

TTTCCCTCCCGGAGATTCCCTAA; JB2-3',

TGAGAGCTGTCTCCTACTATCGATT; VB12-5',

AGTTACCCAGACACCCAGACATGA; JδH (probe),

GGAAGCTTACTTCCAACCTCTTTAGGT; Vδ-5',

GGGGGATCCTGCCTCCTTCTAC; Jδ1-3', AAAAAGCTTACTCAACACGACTGGA.

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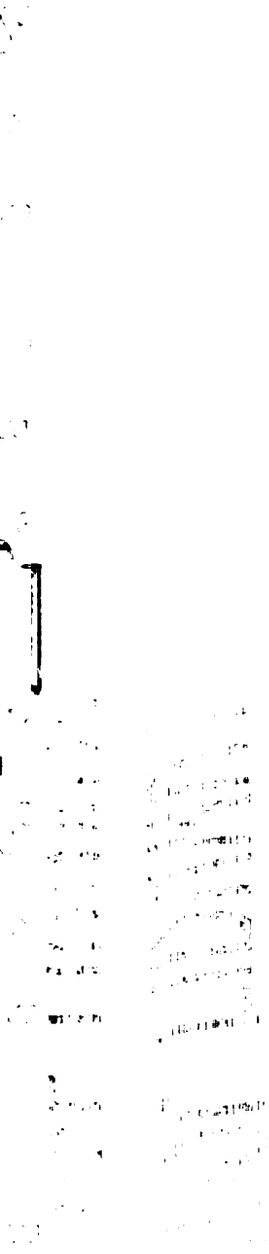

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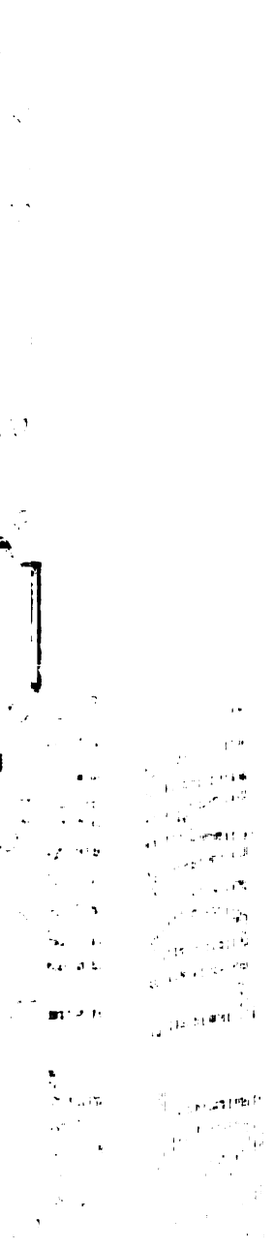


Chapter 4

Concluding remarks

The identification and characterization of a transcription factor are but the first step in defining its role. Understanding the functions of the transcription factor's various domains and determining its complete expression pattern suggests potential targets of transcriptional regulation which must each be individually tested to determine their veracity. We chose to approach the issue of defining the role of LEF-1 during lymphocyte differentiation by analyzing a mouse deficient in the expression of functional LEF-1. Our results have demonstrated that LEF-1 is involved in the regulation of multiple events in lymphocyte differentiation. Some, particularly in the B cell lineage, are not essential for differentiation, and others, as with the B1a cells, are specific to the particular strain of mouse studied. This study reaffirms the previous *in vitro* data detailing the importance of LEF-1 in the regulation of the TCRα enhancer and demonstrates absolutely that LEF-1 participates in normal T cell differentiation. While these data provide some of the answers that we were searching for, many old questions remain and many new ones arose. The following discussion will address these issues and suggest potential experiments.

Identifying new target genes of LEF-1.

Within the B lymphocyte lineage, several targets of regulation are directly suggested from the analysis performed by Tannishtha Reya (BP-1) [Reya and Grosschedl, unpublished results], myself (MHC class II), and Mary O'Riordan (TdT) [O'Riordan and Grosschedl, unpublished results], in that these genes had modified expression levels in the absence of LEF-1. Analysis of the regulatory regions of these genes may provide evidence validating these potential targets, however it is also possible

that LEF-1 affects the expression of the genes indirectly through an as yet undetermined mechanism. To avoid the limitations of sequentially analyzing known lymphocyte-specific genes for dependence on LEF-1 expression, a more fruitful effort would be to differentially screen between LEF-1-deficient and wild type cell populations. For conventional B cells, this would mean isolating the pro-B cell stages B and C from both genotypes. This process is limited by the small number of cells that can be isolated from the bone marrow of the neonatal animals. However, since similar defects are observed in B lymphocytes isolated from adoptive transfers, it should be possible to use these animals to recover larger amounts of cells for this experiment. Another method that could potentially reveal additional targets of LEF-1 regulation would be to perform a cross of the LEF-1-deficient strain with a TCF-4-deficient line. The laboratory of Hans Clevers (reference) has generated such a mouse and our laboratory is currently in collaboration with him to execute this experiment. Since both HMG family members have an overlapping expression pattern in B lymphocyte differentiation, it is possible that the absence of both factors would indicate targets that can be regulated in a redundant manner by both genes.

In the T cell lineage, the removal of the expression of both LEF-1 and TCF-1 is required to illustrate the dependence of T lymphocyte differentiation upon LEF-1. This demonstrates that all of the targets of regulation by LEF-1 in T lymphocytes can be redundantly regulated by TCF-1. Specifically, LEF-1 regulates the transcription of genes important for the $CD25^{+}CD44^{-}$ DN and the $CD8^{+}$ ISP stages of differentiation. During the $CD25^{+}CD44^{-}$ DN stage, signal transduction through the pre-T cell receptor complex is required for maturation. Failure to produce pTα [1], lck [2], RAG-1, RAG-2 [3], or a

successfully rearranged TCRβ product [4] will result in either a complete or incomplete block in differentiation at this stage. Expression of these potential targets was analyzed and none of them were found to be affected by the absence of LEF-1 and TCF-1. Since no identified gene other than LEF-1 and TCF-1 has been shown to be required for the $CD8^+$ ISP T lymphocyte population, we are left with no known candidates to investigate as target genes for LEF-1 in T lymphocyte differentiation. To identify the essential products redundantly regulated by LEF-1 and TCF-1, one approach would again be to use a differential screen of wild type and double-deficient cell populations. Unfortunately, because of the paucity of material available for such an experiment (each *Lef1*$^{-/-}$*Tcf1*$^{-/-}$ FTOC will produce $5x10^3$ $CD8^+$ ISP cells and less than $1x10^3$ $CD25^+CD44^-$ DN cells), acquiring enough cells would be technically difficult. Using a PCR-assisted differential display assay or perhaps a Gene Chip kit (Affymetrix) could allow for the successful isolation of a new target gene, but even these techniques will require a tremendous effort to sort enough source material. The end result, however, should reveal novel essential mechanisms for the differentiation of T lymphocytes.

LEF-1 is required differentially between mouse strains..

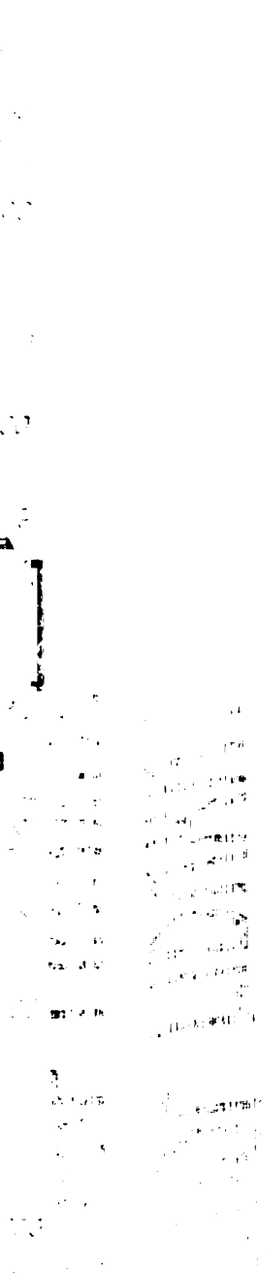
Embryonic stem (ES) cell lines are the essential component of generating targeted disruptions in mice. The ES cell line used by the Grosschedl laboratory is the D3 line that was derived from the 129 Steel substrain 129/SvPas. The initial analysis performed with LEF-1-deficient mice utilized an F1 cross between C57BL/6 and D3 strains. In order to remove variability contributed to our data from the mixed genetic background, we chose to backcross our mice to the C57BL/6 strain. After three or four generation of

backcrossing, we began to notice that the LEF-1-deficient had a younger mortality age as compared to the previous experimental animals. The average lifespan of 10 days and maximum viability of 21 days had both been reduced to an average lifespan of 1 day after birth and a maximum viability of 2 days. While the backcrossing did not affect the previous data collected characterizing *Lef1*$^{-/-}$ T cells or *Lef1*$^{-/-}$ conventional B cells, the ability of LEF-1-deficient lymphocyte precursors to generate CD5^{+} B1 cells was restored. This striking difference led us to backcross the mice to the 129/SvJ strain of mice. This is not the most related substrain of 129 to the D3 line, as 129/SvlmJ would have been a better choice [5]. Despite this, within the first backcross, viability increased to a maximum of four days and by the second backcross, viability was seen up to fifteen days. This simple phenotype of an extended lifespan would immediately suggest that the requirement for LEF-1 differs between the C57BL/6 strain and the D3 embryonic cell line. Determining the cause of the strain difference for the generation of CD5^{+} B1 cell would be another major undertaking as there is no possibility to differentially screen a sorted population of CD5^{+} B1 precursors (there is no identified committed precursor for this lineage other than the multipotential hematopoetic stem cell (HSC)). The most obvious possible explanation, based on the data from T lymphocytes, is that there is another redundant HMG family member active in the CD5^{+} B1 cells in the C57BL/6 strain, but not in the D3 line. However, without a D3 line to test, no exploration of this model is currently possible. Perhaps the only way to address this question correctly is to rederive the D3 strain using the ES cells, and backcrossing the LEF-1-deficiency to this strain. This would provide fetal liver HSCs that could potentially contain a difference in gene expression, providing a cause for the differential requirement for LEF-1.

Defining the relative importance of the functional domains of LEF-1.

LEF-1 has been shown to contain numerous functional domains, including a HMG DNA-binding domain [6], a context-specific activation domain (CAD) [7, 8] and an amino terminal β-catenin interaction domain [9-12]. While the HMG domain is clearly essential for the ability of LEF-1 to regulate transcription, the importance of the other two identified domains depends on the target of regulation. In the case of the TCRα enhancer, while the CAD is essential for normal activity, the β-catenin interaction domain apparently is not necessary [13]. However, on the *Drosphophila Ultrabithorax* promoter, the interaction of β-catenin and murine LEF-1 is required to activate transcription. This ability of LEF-1 to have multiple modes of transcriptional activation opens the question as to how many of the targets of LEF-1 regulation are β-catenin dependent and how many are β-catenin independent. To answer this question, transgenic mice could be created which would express various forms of LEF-1. Among the obvious candidates for this strategy would be constructs bearing the HMG domain alone or with either the CAD or the β-catenin interaction domain. These transgenic mice could then be crossed with LEF-1-deficient mice to determine which of the various mutant forms of LEF-1 are able to restore proper gene expression in the B lymphocytes. To ask the same question of the T lymphocytes, the transgenic mice could be crossed with the double-deficient *Lef1*$^{-/-}$*Tcf1*$^{-/-}$. Assuming that the 129/SvJ backcross is able to restore the CD5^{+} B1 phenotype, these transgenic mice could also be used to determine which domains are important for the generation of this non-conventional B cell population. Members of the

Grosschedl laboratory have already initiated the production of these mice and the results are pending.



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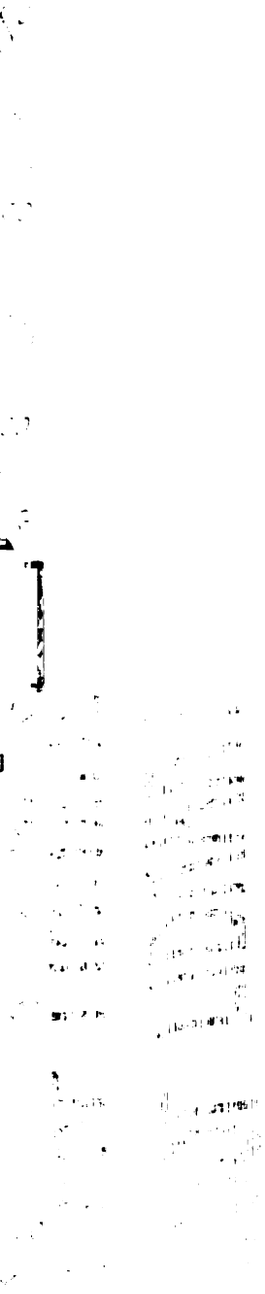

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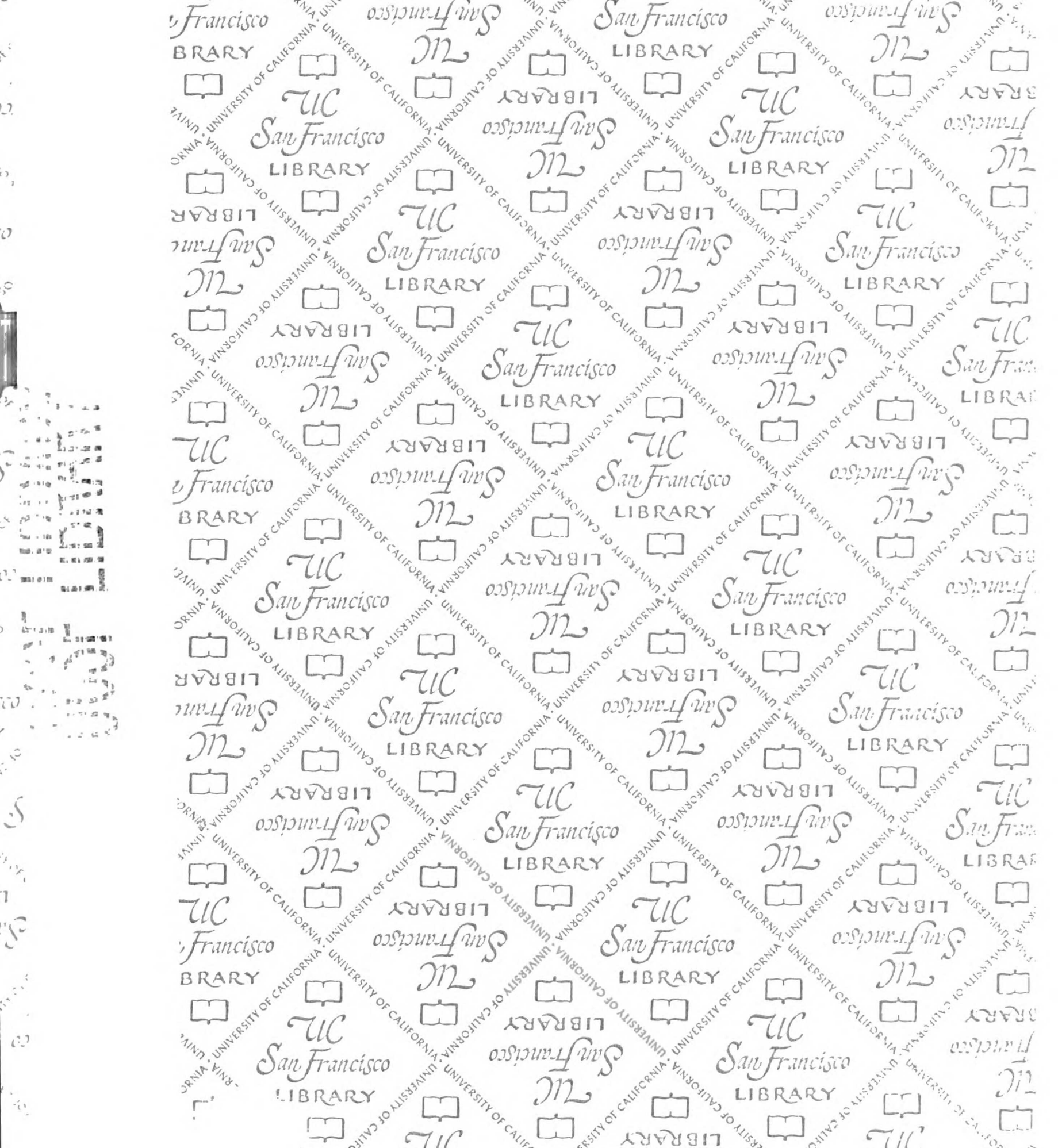
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